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MOLECULAR BASIS OF SKELETAL MUSCLE ATROPHY IN MYOTONIC DYSTROPHY

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Contents

Part I

1. Abstract

Myotonic dystrophy (DM) is an autosomal dominant multisystemic disorder characterized by a variety of multisystemic features including myotonia, muscular dystrophy, cardiac dysfunctions, cataracts and insulin-resistance. DM1 is caused by an expanded (CTG)n in the 3' UTR of the *DMPK* gene, while DM2 is caused by the expansion of a (CCTG)n repeat in the intron 1 of the *CNBP* gene. In both forms, the mutant transcripts accumulate in nuclear foci altering the function of some alternative splicing regulators which are necessary for the physiological processing of mRNAs. However, the downstream pathways by which these RNA binding proteins cause skeletal muscle alteration are not well understood. For these reasons the aim of my PhD project was to analyze the molecular mechanisms behind DM skeletal muscle atrophy. In the first part of my PhD we have performed different studies to better define the molecular pathogenesis of DM. In particular, we have analysed the histopathological and biomolecular features of skeletal muscle biopsies from a cohort of DM1 and DM2 patients presenting different phenotypes. The results indicated that the splicing and muscle pathological alterations observed are related to the clinical DM1 and DM2 phenotype and that CUGBP1 seems to play a role only in DM1, confirming that the molecular pathomechanism of DM is more complex than the one actually suggested. These data were confirmed by the analysis of two different biopsies obtained from 5 DM2 patients that showed that morphological alterations evolve more rapidly over time than the molecular changes suggesting that the molecular mechanisms that drive to skeletal muscle atrophy are still unclear and that these features cannot be explained only by spliceopathy. For all these reasons we decided to analyse DM satellite cells activity *in vitro*. Satellite cells are the muscle fibre precursor cells and our data indicated that both DM1 and DM2 skeletal muscle cells have lower proliferative capability than control myoblasts. Moreover, the premature proliferative growth arrest observed in DM cells appears to be caused by an overexpression of p16 in DM1 muscle cells, while DM2 muscle cells stop dividing with telomeres shorter than controls, suggesting that in these cells the signaling involved in premature senescence depend on a telomere-driven pathway. Finally, we decided to analyze the insulin pathway which is involved in the regulation of skeletal muscle atrophy. Our data have shown that DM1 and DM2 cells exhibit a lower glucose uptake and a lower proteins activation after 10 nM insulin stimulation when compared to controls suggesting that also this pathway could play a role in the molecular mechanisms that drive skeletal muscle atrophy in DM patients. In conclusion, we have shown that the molecular mechanisms behind skeletal muscle atrophy in DM1 and DM2 patients are more complicated than that previously suggested and further analysis are necessary to understand why skeletal muscle atrophy affect mainly type 1 fibres in DM1 patients, while on the contrary it affects selectively type 2 fibres in DM2 patients.

2. State of the Art

2.1 Myotonic Dystrophy

Myotonic dystrophy (DM) is an autosomal dominant multisystemic disorder characterized by a variety of multisystemic features including myotonia, muscular dystrophy, cerebral involvement, cardiac dysfunctions, cataracts and insulin-resistance (Mankodi and Thornton, 2002; Meola and Moxley, 2004). In 1909 Steinert and colleagues first described the first type of myotonic dystrophy (Myotonic Dystrophy type 1 or Steinert's disease) that in 1992 was found to be caused by an expansion of an unstable CTG trinucleotide repeat in the 3' untranslated region (UTR) of the myotonic dystrophy protein kinase gene (*DMPK*) which is located on chromosome 19q13.3 and codes for a myosin kinase expressed in skeletal muscle (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). Subsequently, in 1994, a different multisystemic disorder was described and named myotonic dystrophy type 2 or proximal myotonic myopathy (PROMM). This type of DM was found to be caused by an unstable tetranucleotide repeat expansion, CCTG, in intron 1 of the nucleic acid-binding protein (*CNBP*) gene (previously known as zinc finger 9 protein gene, *ZNF9*) on chromosome 3q21 (Ricker et al., 1994; Liquori et al., 2001).

A population-based genetic screen to determine the true frequency of DM has not yet been performed on a large scale (Thornton 2014). The most ambitious genetic screen to date showed a DM gene frequency of 1 in 1100 among Finnish blood donors, equally divided between DM1 and DM2 (Suominen et al., 2011). A referral center in England found that DM1 was the most common genetic neuromuscular disease, accounting for 29% of the population in a muscle clinic (Norwood et al., 2009). However, other studies estimates that DM1 prevalence in Europe ranges between 1 in 8300 and 1 in 10700 (Magee et al., 1999; Siciliano et al., 2001). Regarding DM2, there are fewer epidemiologic studies of this disease if compared to DM1. The genetic diagnosis of DM1 and DM2 was made with similar frequency at a reference laboratory in Germany, suggesting that the prevalence of the two disorders is similar in northern Europe (Udd et al., 2006). However in the United States clinical experience suggests that DM2 is 5-fold less common than DM1 (Thornton 2014).

Although DM1 and DM2 have similar symptoms, they also present a number of very dissimilar features making them clearly separate diseases (table 1).

Clinical Features	DM1	DM2
General features		
Epidemiology	Widespread	European
Age of onset (years)	0 to adult	8-60
Anticipation	Always present	Exceptional
Congenital form	Present	Absent
Life expectancy	Reduced	Normal range
Core features		
Clinical myotonia	Evident in adult- onset	Present in <50%
EMG myotonia	Always present	Absent or variable in many
Muscle weakness	Disabling at age 50	Onset after age 50-70
Cataracts	Always present	Present in minority
Muscle symptoms		
Facial and jaw weakness	Always present	Usually absent
Bulbar weakness-dysphagia	Always later	Absent
Respiratory muscles weakness	Always later	Exceptional
Distal limb muscle weakness	Always prominent	Only flexor digitorium profundus, rare
Proximal limb muscle weakness	May be absent	Main disability in most patients, late
Sternocleidomastoid weakness	Always prominent	Prominent in few
Myalgic pain	Absent or mild	Most disabling symptom in many
Visible muscle atrophy	Face, temporal, distal hands and legs	Usually absent
Calf hypertrophy	Absent	Present in ≥50%
Systemic features		
Tremors	Absent	Prominent in many
Behavioral change	Early in most	Not apparent
Cognitive disorders	Prominent	Not apparent
Hypersomnia	Prominent	Infrequent
Cardiac arrhythmias	Always present	From absent to severe
Male hypogonadism	Manifest	Subclinical in most
Manifest diabetes	Frequent	Infrequent

Table 1: Comparison of clinical manifestation between DM1 and DM2 (Meola 2013).

The spectrum of DM1 severity extends from lethal effects in infancy to mild, late-onset symptoms. Indeed, DM1 can be an adult-onset multisystem degenerative disorder but it also may affect fetal development and postnatal growth in individuals who carry large expansions. Indeed, patients with DM1 can be divided into four main categories: congenital, childhood-onset, adult-onset and late-onset/asymptomatic (table 2).

Phenotypes	Clinical findings	CTG length	Age of onset
Congenital	Infantile hypotonia Respiratory failure Learning disability Cardiorespiratory complications	> 1000	Birth
Childhood onset	Facial weakness Myotonia Low IQ Conduction defects	50-1000	1-10 years
Adult onset "classic DM1"	Weakness Myotonia Cataracts Conduction defects Insulin resistance Respiratory failure	50-1000	10-30 years
Late onset/ Asymptomatic	Mild myotonia Cataracts	50-100	20-70 years
Pre-mutation	None	38-49	N/A

Table 2: Summary of myotonic dystrophy type 1 phenotypes, clinical findings and CTG length (Meola 2013).

Congenital DM1 (CDM) often presents before birth as polyhydramnios and reduced fetal movements. After delivery, the main features are severe generalized weakness, hypotonia and respiratory compromise that leads to a high mortality from respiratory failure. Surviving infants experience gradual improvement in motor function, since almost all CDM children are able to walk. Another characteristic feature of CDM is the delay in cognitive and motor development and all patients with CDM have learning difficulties and require special need schooling. Moreover, cerebral atrophy and ventricular enlargement are often present at birth (Spranger et al., 1997; Ashizawa 1998). A progressive myopathy and the other main features of the adult classical form of DM1 can be develop in early adulthood in these patients (Joseph et al., 1997). The diagnosis of the DM1 childhood onset form is often missed in affected adolescents or children because of uncharacteristic symptoms for a muscular dystrophy and apparently negative family history (Steyaert et al., 2000). The main features of classic adult-onset DM1 are distal muscle weakness, facial weakness and wasting. Moreover, these patients can develop cardiac dysfunctions such as conduction abnormalities with arrhythmia and conduction blocks contributing significantly to the mortality of the disease (Bassez et al., 2004; Chebel et al., 2005; Montella et al., 2005; Dello Russo et al., 2006). Another characteristic feature of adult DM1 is posterior subcapsular cataracts (Garrot et al., 2004). Finally, endocrine abnormalities are common in these patients and include testicular atrophy, hypotestosteronism, insulin resistance with usually mild type-2 diabetes (Meola 2000). In late-onset or asymptomatic DM1 patients myotonia, weakness and excessive daytime sleepiness are rarely present (Meola and Cardani, 2014).

In DM2 there are no distinct clinical subgroups although initially different phenotypes of DM2 with proximal muscle weakness were described: DM2/Proximal Myotonic Myopathy (PROMM) and Proximal Myotonic Dystrophy (PDM) (Ricker et al., 1994; Thornton et al., 1994; Udd et al., 1997). PROMM typically appears in adult life and has variable manifestations, such as early-onset cataracts (younger than 50 years), myotonia, muscle pain and weakness (Thornton et al., 1994; Ricker et al., 1995; Meola et al., 1996; Udd et al., 1997; Day et al., 1999; Ranum et al., 1998; Ashizawa et al., 2000; Meola 2000; Liquori et al., 2001; Moxley et al., 2002; Day et al., 2003; Schoser et al., 2004). Myotonia is often less apparent in DM2 compared with patients with DM1 and, in cases of late-onset DM2, myotonia may only appear on electromyographic testing after examination of several muscles (Meola 2000). The cataracts in DM2 have an appearance identical to that observed in DM1, while cardiac problems appear to be less severe and frequent in patients with DM2 than in patients with DM1 (Meola et al., 2002; Flachenecker et al., 2003; Sansone et al., 2013). The type of cognitive impairment that occurs in DM2 is similar but less severe than that of DM1. On the contrary, some endocrine dysfunction such as hypogonadism and glucose intolerance may also occur and worsen over time in DM2 (Thornton et al., 1994; Meola et al., 1999; Savkur et al., 2001; Day et al., 2003; Meola et al., 2003; Savkur et al., 2004). PDM patients show many features similar to those found in PROMM, including proximal muscle weakness, cataracts, and electrophysiologically detectable myotonia. However they present some features not present in PROMM, such as pronounced dystrophic–atrophic changes in the proximal muscles and late-onset progressive deafness (Udd et al., 1997).

The most important discrepancy between DM1 and DM2 is absence of a congenital form in DM2 (Day et al., 2003; Udd et al., 2003). In patients affected by DM1 the repeat size range is from 50 to 4000 CTG and is nearly always associated with symptomatic disease. Healthy individuals have between 5 and 37 CTG repeats while repeat lengths of 38–50 are considered premutation alleles, whereas 51–100 repeats are protomutations, both of which show increased instability toward expansion. Patients with premutations or protomutations are asymptomatic or present few mild symptoms, such as cataracts, but are at risk of having children with larger, pathologically expanded repeats (Thornton et al., 1994). The DM1 mutation length of 2000 repeats causes the congenital form of the disease (Ashizawa et al., 2000; Schoser et al., 2010). Since the size of the CTG repeat appears to increase over time in the same individual and across generation, children may inherit considerably longer CTG repeats than those present in the transmitting parent leading to a phenomenon known as genetic anticipation in which disease severity increases and/or age of onset of disease decreases from one generation to the next. However, the CTG repeat size does not always increase in successive generations of DM families. Intergenerational contraction of CTG repeats, a decrease in the CTG repeat size during transmission from parents to child, can also occur in about

6.4% of transmissions, most frequently during paternal transmissions (10%) (Ashizawa et al., 1994; Puymirat et al., 2009). In DM2, the size of the normal (CCTG)n expansion is below 30 repeats, while the range of expansion sizes in DM2 patients is huge (Bachinski et al., 2009). The smallest reported mutations vary between 55 and 75 CCTG and the largest expansions have been measured to be up about 11000 repeats (Liquori et al., 2001; Bachinski et al., 2009). The expanded DM2 alleles show marked somatic instability, with significant increases in length over time (Liquori et al., 2001; Day et al., 2003). Since in DM2 the mutation usually contracts in the next generation being shorter in the children, this type of DM is not characterized by genetic anticipation and the presence of the congenital phenotype (Day et al., 2003; Udd et al., 2003). Moreover a large proportion of DM2 patients may be undiagnosed. Indeed, recent studies indicate that the co-segregation of heterozygous recessive CLCN1 mutations in DM2 patients is higher than expected and modifies the DM2 phenotype (Suominen et al., 2008; Cardani et al., 2012). More recently, Bugiardini et al. have identified the first case of a DM2 patient with a concomitant mutation on SCN4A gene that codes for Nav1.4 voltage gate sodium channel (VGSC) expressed in muscle (Catterall et al., 2005; Bugiardini et al., 2015). Thus the CLCN1 or SCN4A mutations may contribute to exaggerating the DM2 phenotype and these patients could be more easily identified and diagnosed than DM2 patients without the modifier allele.

2.2 Pathogenetic mechanism

As described above, DM1 is caused by an expanded (CTG)n in the 3' untranslated region of *DMPK* gene, while DM2 is caused by an expanded (CCTG)n in the intron 1 of *CNBP* gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992; Ricker et al., 1994; Liquori et al., 2001). Although genetically distinct, DM1 and DM2 share a common pathogenic mechanism. Experimental evidence supports a "RNA gain-of-function" mechanism in which expanded CUG/CCUG-containing transcripts accumulate in the cell nuclei as ribonuclear inclusions and are responsible for the pathologic features common to both disorders (figure 1). Indeed, the mutant RNAs form imperfect double-stranded structure which lead to the deregulation of several RNA binding factors, including the muscleblind-like proteins (MBNLs), CUGBP1, hnRNP H and Staufen1 proteins (Timchenko et al., 1996; Philips et al., 1998; Miller et al., 2000; Kanadia et al., 2003; Ho et al., 2004; Paul et al., 2006; Ravel-Chapuis et al., 2012). The MBNL proteins appear to play a prominent role in DM pathogenesis since each of the three MBNL isoforms (MBNL1, MBNL2 and MBNL3) are sequestered by CUG RNAs in the cell nuclei (Miller et al., 2000; Faradei et al., 2002). Moreover Mbnl1 and Mbnl2 knockout mice recapitulate multiple features of adult-onset DM (Kanadia et al., 2003, 2003). Interestingly, while Mbnl1 knockout mice develop the muscle, eye, and RNA splicing abnormalities that are characteristic of DM1 disease and show

modest effects on alternative splicing regulation in the brain, the loss of Mbnl2 leads to widespread changes in postnatal splicing patterns in the brain but not in skeletal muscle (Kanadia et al., 2003; Suenaga et al., 2012). Even if nothing is known about the functions of MBNL3 *in vivo*, *in vitro* studies show that MBNL3 acts as an antagonist of myogenesis possibly by maintaining myoblasts in a proliferative state. Moreover Mbnl3 isoform knockout mice show age-dependent impairment of adult muscle regeneration suggesting that Mbnl3 inhibition by toxic RNA expression may be a contributing factor to the progressive skeletal muscle weakness and wasting characteristic of DM (Squillace et al., 2002; Lee et al., 2008; Poulos et al., 2013). CUGBP1, a member of the family of CELF (CUGBP, Elav-like family) proteins, is a regulator of alternative splicing and of mRNA translation and stability (Timchenko et al., 2005; Barreau et al., 2006; Huichalaf et al., 2009, 2010; Lee et al., 2010). CUGBP1 does not colocalize with ribonuclear foci in DM1 cells, however this protein was found to be overexpressed in DM1 myoblasts, skeletal muscle and heart tissues due to PKC-mediated hyperphosphorylation and subsequent protein stabilization and upregulation (Timchenko et al., 1996; Miller et al., 2000; Savkur et al., 2001; Fardaei et al., 2002; Mankodi et al., 2003; Dansithong et al., 2005; Kuyumcu-Martinez et al., 2007). On the contrary the role of CUGBP1 in DM2 is particularly intriguing with contradictory results being reported. Timchenko and colleagues reported an increase of CUGBP1 in DM2 cultured myoblasts and muscle biopsies analyzing cytoplasmic extracts. Moreover they reported that expression of pure RNA CCUG repeats in normal human myoblasts, in C2C12 cells and in a DM2 mouse model also increased levels of CUGBP1 (Salisbury et al., 2009). On the contrary, in two different reports, the analysis of total cellular extract from DM2 cultured myoblasts and from muscle biopsies of DM2 patients did not show differences in CUGBP1 levels (Lin et al., 2006; Pelletier et al., 2009). Other splicing factors involved in early phases of pre-mRNA processing have been found to be altered in DM pathologies confirming that a general alteration of pre-mRNA processing could be at the basis of DM phenotype. Indeed an elevation of the steady-state level of hnRNPH has been observed in both DM1 and DM2 myoblasts (Fakan 1994; Paul et al., 2006; Perdoni et al., 2009). Staufen1 is another alternative splicing regulator that has been found to be increased in skeletal muscle from DM1 mouse models and patients. Interestingly, Staufen1 up-regulation might have a protective role in the DM1 pathology since it appears that the increase in Staufen1 may be a compensatory mechanism used by muscle fibres to reduce and/or delay the pathologic effects caused by MBNL1 sequestration and CUGBP1 up-regulation (Ravel-Chapuis et al., 2012). Thus, the misregulation of alternative splicing clearly plays a central role in the development of important DM symptoms. Indeed , among the symptoms of DM, myotonia, insulin resistance and cardiac problems seem to be correlated with the disruption of the alternative splicing of some specific proteins. Indeed, it has been reported that an alteration in the insulin receptor (IR) splicing can lead to insulin insensitivity and a

predisposition to diabetes, an alteration in the muscle chloride channel (CLCN1) can lead to myotonia, while an alteration in skeletal muscle calcium ATPase SERCA may contribute to the Ca^{2+} homeostasis impairment that characterise DM muscle cells (Savkur et al., 2001; 2004; Kimura et al., 2005).

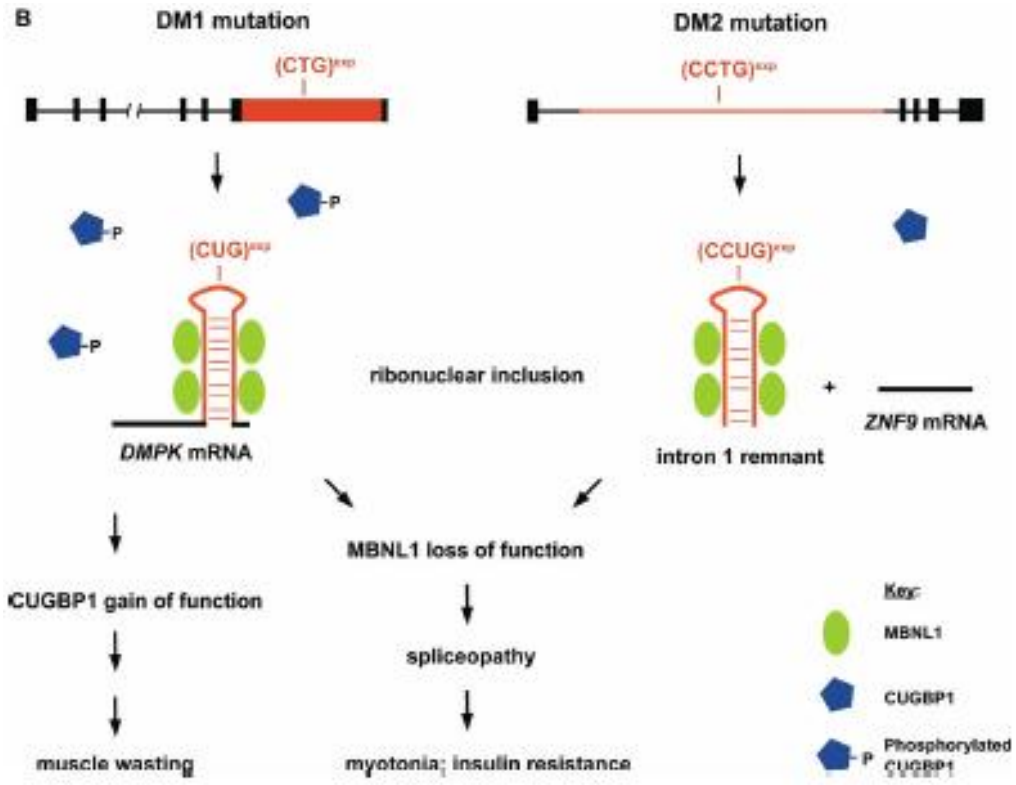

Figure 1: "RNA gain of function" model in DM1 and DM2 (Wheeler 2008).

However, there is no direct evidence of a cause–effect relationship between symptoms and missplicing and it seems that spliceopathy may not fully explain the multisystemic disease spectrum. Indeed, Bachinski and collaborators performed global array-based expression and splicing profiling on a large number of DM and non-DM neuromuscular patients and found that DM1 and DM2 skeletal muscles were essentially identical to each other for both expression and splicing. Moreover the authors found no evidence for widespread missplicing as a DM-specific pathomechanism in skeletal muscle since most expression and splicing changes were shared between multiple muscular dystrophies, as previously suggested (Bachinski et al., 2010; 2014; Orengo et al., 2011). This evidence suggests that splicing changes may be a much more general phenomenon of muscle disease and can be secondary to muscle regeneration (Bachinski et al., 2010; Orengo et al., 2011). All these studies strongly suggest that DM molecular pathogenesis may be more complex involving changes in gene expression and translation efficiency, non-conventional translation and micro-RNA (miRNA) deregulation (figure 2). The effects of repeat expansion on

gene expression were revealed by microarray analysis of DM1 and DM2 muscle biopsies and confirmed by the mRNA profiling of transgenic mice expressing non-coding CUG repeats in skeletal muscle (Botta et al., 2007). Interestingly, most of the changes detected were also found in Mbnl1 knock-out mice, indicating that transcriptional deregulation results partially from MBNL1 loss of function (Osborne et al., 2009). Altered gene expression may also result from a direct effect of the repeat expansion on transcription factors. Indeed, expanded DMPK transcripts bind SP1 and RARγ in DM1 myocytes, reducing their availability and the expression of target transcripts (Ebralidze et al., 2004). Moreover NKX2-5 upregulation in DM1 skeletal muscle and heart affects the expression of downstream genes, possibly contributing to the muscle regenerative defects and cardiac conduction abnormalities (Yadava et al., 2008). The mislocalization of SHARP (SMART/HDAC1- associated repressor protein) in the cytoplasm in DM1 myoblasts may alter the steady-state levels of a set of RNA transcripts implicated in muscle development and function (Dansithong et al., 2011). The absence of co-localization of transcription factors with CUG-containing nuclear foci does not exclude a pathogenic role. Indeed, a fraction of expanded DMPK transcripts does not aggregate and remains soluble in DM1 cells, interacting with splicing regulators and transcription factors and thus contributing to pathology (Junghans 2009). A novel molecular mechanism that may contribute to the pathogenesis of myotonic dystrophies has been described by Ranum's group (Zu et al., 2013). RNA transcripts containing expanded CAG repeats can be translated in the absence of a starting ATG and this non-canonical translation, called Repeat Associated Non-ATG translation (RAN-translation) occurs across expanded CAG repeats in all reading frames (CAG, AGC, and GCA) to produce homopolymeric proteins of long polyglutamine, polyserine, and polyalanine tracts. RAN translation across human spinocerebellar ataxia type 8 (SCA8) and myotonic dystrophy type 1 (DM1) CAG expansion transcripts results in the accumulation of SCA8 polyalanine and DM1 polyglutamine expansion proteins in SCA8 and DM1 mouse models and human tissue (Zu et al., 2011). Antibodies developed specifically against DM1 polyGln proteins, detect polyGln nuclear aggregates in DM1 mouse tissues and DM1 patient cardiac myocytes, leukocytes, and myoblasts not detectable in control tissues. RAN-translation products appear to be toxic to cells and may contribute to DM1 pathology. More recently RAN translation has been found to occur across intronic DM2 CCUG transcripts and that these transcripts produce a tetra-repeat expansion protein with a repeating Leu-Pro-Ala-Cys (LPAC) motif. Moreover, an LPAC antibody shows strong immunostaining in human DM2 autopsy brain but not in controls. Immunostaining has been observed in neurons, astrocytes and glia in frontal cortex, hippocampus and basal ganglia. These data suggest that RAN translation may be common to both DM1 and DM2 and that RAN proteins may be responsible for some of the CNS features of DM (Zu et al., 2013). miRNAs are small non-coding RNA modulating gene expression at posttranscriptional

level and their expression and intracellular distribution are deregulated in many human diseases, including muscular dystrophies (Eisenberg et al., 2009; Greco et al., 2009, 2012; Gambardella et al., 2010; Perbellini et al., 2011). Both in DM1 and DM2 it has been demonstrated that the highly regulated pathways of miRNA is altered in skeletal muscle potentially contributing to DM pathogenetic mechanisms (Gambardella et al., 2010; Perbellini et al., 2011; Greco et al., 2012). Interestingly, miR-1 downregulation in DM1 and DM2 hearts is mediated by the functional depletion of MBNL1, which affects the misprocessing of pre-miR-1 (Rau et al., 2011). Whether MBNL1 regulates the processing of other miRNAs remains to be determined. DM-associated miRNA deregulation alters the expression of target transcripts, possibly contributing to disease pathology (Perbellini et al., 2011; Rau et al., 2011). Interestingly, Perfetti et al. identify a signature of miRNA deregulated in peripheral blood plasma from DM1 patients . In particular one specific miRNA, miR-133a, clearly correlates with muscle strength measurement and increased in patients with higher MIRS score, potentially reflecting disease severity (Perfetti et al., 2014). Finally, in DM1 cells, CUG-containing RNAs form imperfect hairpin structures, which can be cleaved by the RNA interference (RNAi) machinery to generate CUG-containing small interference RNAs (siRNAs) that are capable of binding complementary sequences in target mRNAs, possibly interfering with their expression and contributing to disease pathogenesis (Krol et al., 2007).

The understanding of DM pathogenetic mechanism is improving fast, nevertheless there are some key aspects of the disease that deserve further investigation. Indeed the neurological manifestations of DM, the differences between DM1 and DM2 clinical features such as the presence of a congenital phenotype in DM1 and histopatholgical alterations in skeletal muscle does not still have a definitive molecular explanation.

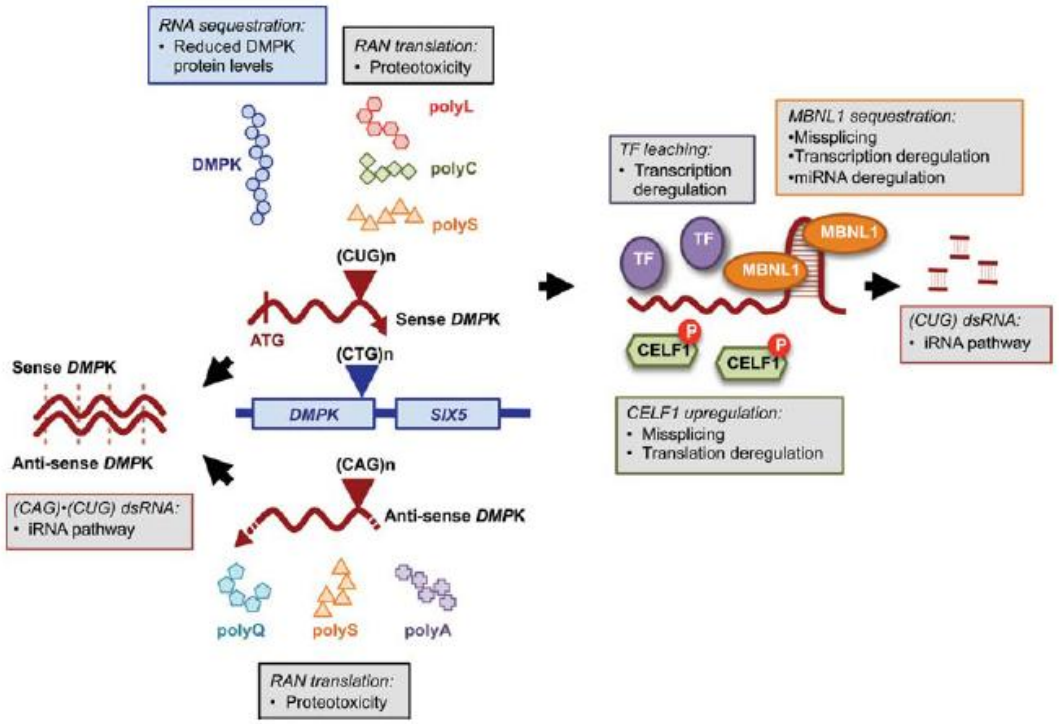

Figure 2: Molecular pathogenesis of DM1: mechanisms of RNA toxicity, spliceopathy, deregulation of gene expression and proteotoxicity (Sicot et al., 2011).

2.3. Skeletal muscle histopathology in DM1 and DM2

The histological features of skeletal muscle biopsy in DM1 and DM2 are very similar, and sufficiently characteristic that a diagnosis of DM can be suggested based on muscle biopsy alone (table 3) (Day et al., 2003; Schoser et al., 2004).

Histopathological findings	DM1	DM2
Fiber size variation	+++	+++
Internal nuclei	**+++**	**+++** more in type 2 fibers
Type 1 fiber atrophy	++	-
Type 2 fiber atrophy	+	++
Type 2 fiber hypertrophy	-	+
Nuclear clump fibers	**+** at advanced stages only	**+++** more in advanced stages
Atrophic fibers (diam. ≤ 6µm)	**±** type 1 and type 2 fibers at advanced stages only	**+++** type 2 fibers
Ring fibers	++	+
Sarcoplasmic masses	++	±
Fibrosis	**+++** at late stages only	**++** at late stages only
Fatty replacement	**+++** at late stages only	**++** at late stages only

Table 3: Muscle histopathology in DM1 and DM2. +++ present in >75% of biopsies; ++ present in 20-50% of biopsies; + present in 10-24% of biopsies; ± occasionally present; - absent (Meola 2013).

In both diseases, affected muscles present an increase in the number of central nuclei and a high variability in fibres diameters that commonly ranges from less than 10 μm to greater than 100 μm. Moreover, in both disease, basophilic regenerating fibres, splitting fibres, fibrosis and adipose deposition can occur in relation to the extent of muscle involvement. Ring finger fibres and sarcoplasmic masses are generally more frequent in DM1 muscle biopsy. However, the comparison of muscle biopsy findings in classic DM1 with those in DM2 has indicated that specific features are present in both diseases. The presence of severely atrophic fibres with pyknotic nuclear clumps are frequently found in DM2 biopsies also before the occurrence of muscle weakness, while on the contrary in DM1 this feature is present only in end-stage muscle biopsy (Vihola et al., 2010). Moreover, a predominant type 2 fibre atrophy in DM2 muscle biopsies has been described, while on the contrary in DM1 biopsies fibre atrophy seems to affect mainly type 1 fibres (Vihola et al., 2003; Schoser et al., 2004; Bassez et al., 2008; Pisani et al., 2008). In DM2 muscle biopsy central nucleation selectively affects type 2 fibres and the atrophic nuclear clumps express fast myosin isoform (type 2 fibre) indicating that DM2 is predominantly a disease of type 2 myofibres (Bassez et al., 2008) (figure 3).

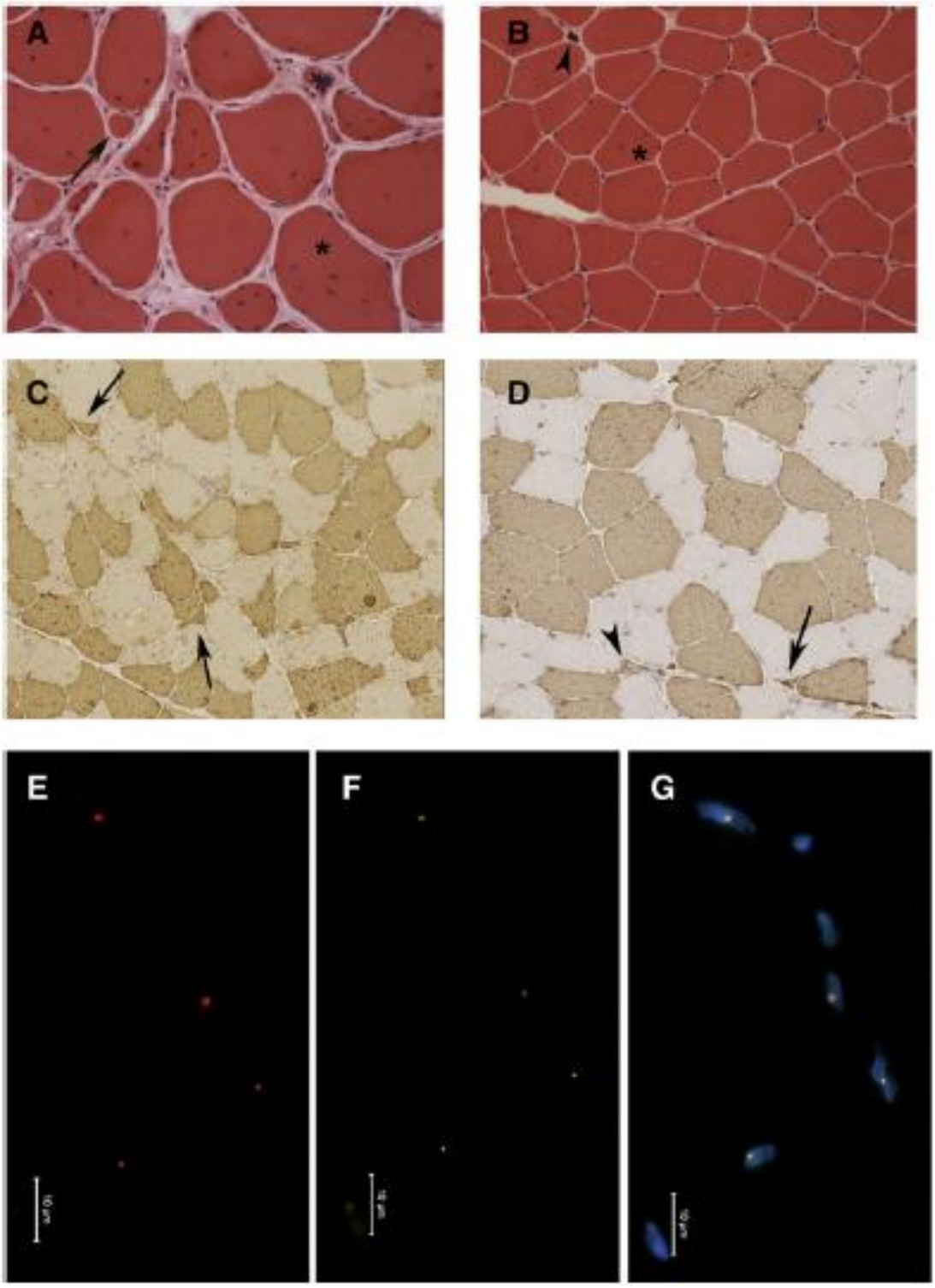

Figure 3: Panel showing muscle histology in DM1 and DM2. A) Hematoxylin & Eosin stained transversal sections of DM1 muscle biopsy presenting a severe fibrosis, fibre size variation (arrow) and central nuclei (asterisk). B) Hematoxylin & Eosin stained transversal sections of DM2 muscle biopsy with fibre size variation, central nuclei and nuclear clump (arrowhead). C) Slow myosin (MHCslow) stained section of DM1 muscle biopsy. The population of atrophic fibres (dark brown) is preferentially type 1 fibres (arrows). D) Fast myosin (MHCFast) stained section of DM1 muscle biopsy. Type 2 fibres (dark brown) are predominantly affected in DM2 muscle. E-G) Fluorescence in situ hybridization (FISH) (E, red spots) in combination with MBNL1-immunofluorescence (F, green sposts) on DM2 muscle biopsy. (Meola and Cardani, 2014).

2.4 Molecular pathways associated with skeletal muscle atrophy in DM1 and DM2

To date there are no definitive explanations for the histopathological alterations observed in DM skeletal muscle.

Since the discover of the DM1 genetic locus, several mouse models have been generated for examination of the molecular basis of DM1 histopathology. DM1 mouse models designed to study DM1 muscle pathology can be divided in two different groups: the first group addresses the role of the accumulation of long CUG, while the second group includes models designed to elucidate the role of the RNA binding proteins CUGBP1 and MBNL1 (Mankodi et al., 2000; Seznec et al., 2001; Kanadia et al., 2003; Timchenkoet al., 2004; Ho et al., 2005; Orengoet al., 2008; Ward et al., 2010). The first transgenic mouse model that demonstrated the crucial role of CUG RNA in DM1 muscle pathology expressed 250 CUG repeats in the 3'-UTR of skeletal muscle actin gene (HSA^{LR} mice) (Mankodi et al., 2000). These mice developed myotonia and skeletal muscle myopathy similar to the muscle histopathology observed in DM1 patients. Indeed, skeletal muscles of these mice showed an increase in the number of central nuclei and variability in fibres size. However, initial studies did not reveal overt muscle atrophy in adult HSA^{LR} mice, while recent analysis showed that the total number of myofibres is reduced in gastrocnemius of six-month-old mice in at least some mouse lines of this model (Mankodi et al., 2000; Jones et al., 2012). A similar effect of mutant CUG repeats on muscle pathology was observed in a DM1 mouse model that expresses the entire human *DMPK* gene containing 300 expanded CTG repeats (DM300 mice) (Seznec et al., 2001). Also these mice developed myotonia and skeletal muscle myopathy similar to the one observed in DM1 patients with central nuclei, increased variability in fibre size and focal areas of regeration-degeneration that express the neonatal myosin heavy chain. Moreover, skeletal muscles of these mice show an increased number of atrophic fibres with specific atrophy of type 1 fibres and whole body weight was reduced even at a young age (Seznec et al., 2001). The same mice with a bigger CTG expansion (550 CTG, DM550) developed progressive age-dependent muscle weakness and wasting associated with reduced muscle mass and fibre diameter (Vignaud et al., 2010). The worst effect of mutant CUG repeats on muscle pathology was observed in the DM1 inducible mouse model expressing a mutant 3'-UTR of *DMPK* containing a 960 CUG repeats under the control of a skeletal muscle promoter. These mice were characterised by a severe muscle wasting that reduced muscle size. Moreover, skeletal muscles of these mice show an increase in small sized fibres, fibrosis, myofibre degeneration, and a reduction in performance on the treadmill (Orengo et al., 2008). The analyses of these DM1 mouse models clearly show that muscle wasting in DM1 is caused by CTG repeats, but however a high variability dependent on the length of CTG expansions and the genomic environment of CTG repeats was observed. Although it is well established that expanded CUG repeats are responsible for the myofibre loss in DM1, the downstream pathways by

which the mutant CUG repeats cause muscle wasting are not well understood. Since it's known that the mutant CUG repeats misregulate mainly two RNA-binding proteins, CUGBP1 and MBNL1, abnormal functioning of these proteins could cause muscle atrophy in DM1 (Milleret al., 2000; Philips et al., 1998; Timchenko et al., 1996). Indeed, mouse models with increased expression of CUGBP1 revealed the crucial role of this protein in muscle development and function. Elevation of CUGBP1 expression in traditional transgenic mouse models interferes with muscle development, causing a delay in myogenesis consistent with the muscle abnormalities observed in congenital DM1 patients (Ho et al., 2005; Timchenko et al., 2004). The muscle of mice with deleted MBNL1 shows myotonia, split fibres, and an increase in central nuclei (Kanadia et al., 2003). MBNL1 and CUGBP1 are not only two splicing regulators. Indeed, recent reports showed that MBNL1 is also involved in control of destabilization of some mRNAs, in miR-1 processing, and in regulating localization of mRNAs (Rau et al., 2011; Masuda et al., 2012; Wang et al., 2012). CUGBP1 is a multifunctional protein that functions in the nuclei and cytoplasm and regulates splicing, stability, and translation of RNAs (Charlet et al., 2002; Lee et al.,2010; Moraes et al., 2006; Paillard et al., 1998; Savkur et al., 2001;Timchenko et al., 2005, 2006; Vlasova et al., 2008; Zhang et al.,2008). MBNL1 and CUGBP1 have antagonistic splicing activities, and their activity is crucial for the regulation of spliced isoforms during normal skeletal muscle and heart development (Ranum and Cooper, 2006). In DM1, a reduction in MBNL1 splicing activity and increase in CUGBP1 splicing activity results in accumulation of embryonic-specific transcripts and their protein products in adult muscle, affecting muscle function and leading to muscle atrophy. Indeed, several mRNAs that play a critical role in normal muscle function show abnormal splicing patterns in DM1, including mRNAs encoding a skeletal muscle chloride ion channel 1 (Clcn1), sarcoplasmic/endoplasmic reticulum Ca^{2+}ATP-ase (SERCA1), a protein localized to the Z line (Cipher), a protein involved in the T-tubule formation (BIN1), Ca^{2+} release channel or Ryanodine Receptor 1 (RyR1), and L-type Ca^{2+}channel and voltage sensor (Cav1.1) (Charlet et al.,2002; Mankodi et al., 2002; Kimura et al., 2005; Kanadia et al., 2006; Fugier et al., 2011; Tang et al.,2012). Missregulation of SERCA1 and RyR1 in DM1 might affect Ca^{2+} homeostasis, leading to the activation of Ca^{2+}-dependent proteases such as calpain and causing muscle atrophy. Abnormalities of BIN1 splicing might disrupt formation of T-tubules resulting in muscle under-development and weakness. The Cav1.1 channel plays a crucial role in the regulation of excitation–contraction coupling (Tanget al., 2002). Even if comparison of splicing patterns in the muscle of HSA^{LR} mice with those in mice with deleted MBNL1 shows similar splicing abnormalities, MBNL1 knock out mice do not show evident muscle wasting suggesting that other members of MBNL family (MBNL2 or MBNL3) might compensate for the lack of MBNL1 (Osborne et al., 2009, Kanadia et al., 2003). However, a critical difference between MBNL1 KO mice and CUG inducible mice is that the MBNL1 KO mice have normal

levels of CUGBP1, whereas CUGBP1 expression is elevated in the inducible DM1 model suggesting that the elevated CUGBP1 could be responsible for muscle wasting in DM1 mouse models (Orengo et al., 2008). Indeed, the analysis of alternative splicing pattern show that in the inducible CUG mouse model present several mRNAs that are mispliced but that have a normal splicing pattern in MBNL1 KO mice. These mRNAs include those encoding Ankyrin 2 (Ank2), F actin capping protein beta subunit (Capzb), and fragile X mental retardation syndrome-associated protein (Fxr1) (Orengoet al., 2008). Ank2 mediates binding of membrane proteins to the cytoskeleton and Capzb is associated with filament growth. Fxr1 is a member of the fragile X family of RNA-binding proteins that regulate RNA processing on several levels and its deletion in mice causes muscle loss suggesting that its alteration in DM1 cells could reduce proteins synthesis in DM1 cells leading to skeletal muscle atrophy (Mientjes et al., 2004; Whitman et al., 2011). In agreement with this suggestion, the CUGBP1 mouse model developed myofibre atrophy accompanied by a reduction in the weight, increased inflammatory infiltrates and fibre degeneration (Ward et al., 2010). Although mice with elevated CUGBP1 expression develop muscle atrophy, the contribution of the splicing activity of CUGBP1 to this feature of the disease remains to be determined. A recent study using a mouse model that expresses a nuclear dominant-negative form of CUGBP1 showed that these mice have disrupted splicing without muscle atrophy, suggesting that abnormal cytoplasmic functions of CUGBP1 might contribute to muscle atrophy in DM1 (Berger et al., 2011). Indeed, in DM1 muscles an increase in the expression of CUGBP1 un-phosphorylated at Ser302 could repress protein translation in stress granules (SGs) leading to muscle wasting (Huichalaf et al., 2010). It has been observed that expanded CUG repeats reduce phosphorylation of Ser302 through a reduction in cyclin D3 due to elevated levels of active GSK3β kinase (Salisbury et al., 2008; Jones et al., 2012). Interestingly the inhibition of GSK3β in HSA^{LR} mice corrected CUGBP1 translational activity and increased the number of the activate myogenic satellite cells (Jones et al., 2012). Satellite cells are the skeletal muscle precursor cells and provide the potential for both pre and post-natal growth of skeletal muscle and for its regeneration following injury (Bischoff et al., 1994; Relaix et al., 2012). In normal muscles, satellite cells are quiescently located between the sarcolemma and the basal lamina of mature myofibres and following injury they become activated and then proliferate and fuse into myotubes to regenerate or repair muscle fibres (Cooper et al., 1999; Renault et al., 2002). It is known that the regenerative capacity of skeletal muscle depends on the number of progenitor cells which declines with age in humans and on their activation, proliferation and differentiation potential (Bodnar et al., 1998; Renault et al., 2002; Wright et al., 2002). The proliferative potential of human satellite cells decreases during postnatal muscle growth due to the replicative senescence that could affect both the regeneration process and the maintenance of muscle mass, since the differentiation program of senescent

myogenic precursor cells would become defective due to impaired myogenesis (Renault et al., 2002; Wright et al., 2002). Indeed, Bigot et al. (2008) observed that senescent myoblasts are still able to fuse and form myotubes, yet significantly smaller and with a significant reduction in the number of nuclei per myotube and in the fusion index, compared with those obtained from young myoblasts (Bigot et al., 2008). In a recent report, it has been demonstrated that satellite cells isolated from DM1 patients reached the proliferative senescence when their mean telomere lengths were longer than those observed in passage-matched control cells indicating that DM1 cells reach proliferative arrest prematurely, independently of telomere shortening (Thornell et al., 2009). It appears that the treatment with GSK3 inhibitors in 6-month-old HSA^{LR} mice increase the number of myogenic satellite cells that is accompanied by the reduction of muscle histopathology and improvement of the grip strength (Jones et al.,2012). However the pathways by which GSK3 inhibitors increase the number of activated satellite cells are still not known.

In contrast to DM1, less is known about the molecular mechanisms that induce muscle atrophy in DM2. Indeed the role of MBNL1 and CUGBP1 in muscle atrophy in DM2 has been poorly investigated. Since it is known that CCUG repeat expansions in DM2 are longer than CUG expansions in DM1, it is expected that DM2 expansions will have a greater inhibitory effect on MBNL1 splicing (Mankodi et al., 2001). However, muscle atrophy in DM2 muscle is milder than that in DM1, suggesting that MBNL1 sequestration and a reduction of MBNL1 splicing activity in DM2 cells might play only a partial role in DM2 muscle atrophy. Also the contribution of CUGBP1 to DM2 requires additional investigation because, as mentioned above, contradictory results about its expression has been reported (Lin et al., 2006; Pelletier et al., 2009; Salisbury et al., 2009). Comparison of DM1 and DM2 myoblast cell culture models showed that levels of active CUGBP1-eIF2 complexes are reduced during differentiation of DM1 myoblasts, whereas the amounts of these complexes are normal in DM2 myotubes suggesting that the regulation of CUGBP1 activity in DM1 and DM2 muscles might be different (Schoser and Timchenko, 2010). Since the discover of the DM2 genetic locus, several studies have been performed to define the possible role of ZNF9/CNBP protein in muscle atrophy and weakness in DM2. Indeed, ZNF9/CNBP plays a key role in the translational control of mRNAs containing 5'-terminal oligopyrimidine (5'TOP) tracts that encode proteins of the translational apparatus including ribosomal proteins and elongation factors (Pellizzoni et al., 1997;Huichalaf et al., 2009; Meyuhas, 2000). DM2 muscle biopsies have shown reduced levels of several TOP proteins leading to a reduction in the rate of global protein synthesis, which might explain the muscle atrophy in DM2 (Huichalaf et al., 2009). The role of ZNF9/CNBP in muscle atrophy is supported by the muscle phenotype in ZNF9 KO mice, which includes fibre size variability and weakness similar to those observed in DM2 patients (Chen et al., 2007). Two recent reports showed that ZNF9/CNBP levels are reduced in DM2 muscle biopsies,

although other authors reported that some DM2 patients have normal levels of this proteins (Botta et al., 2006; Salisbury et al.,2009; Raheem et al., 2010). It is known that muscle atrophy can occur due to a misbalance between protein synthesis and protein degradation and interestingly it has been shown that proteasome activity in DM2 muscle cells is affected, possibly as a result of the binding of RNA CCUG repeats to multiprotein complexes containing the 20S proteasome (Salisbury et al., 2009). Proteomic analysis of DM2 myotubes indicated that these cells have an alteration in the ubiquitin proteasome system that suggests an increase in proteasomal activity in DM2 myotubes that leads to a dysfunction of protein degradation (Rusconi et al., 2010).

3. Aim of the Project

Myotonic dystrophy (DM) is an autosomal dominant multisystemic disorder characterized by a variety of multisystemic features including myotonia, muscular dystrophy, cerebral involvement, cardiac dysfunctions, cataracts and insulin-resistance (Mankodi and Thornton, 2002; Meola and Moxley, 2004). Myotonic dystrophy type 1 (DM1) is caused by an expanded (CTG)n in the 3' untranslated region of *DMPK* gene, while a second form (DM2) is caused by an expanded (CCTG)n in the intron 1 of *CNBP* gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992; Ricker et al., 1994; Liquori et al., 2001). In both forms, the CUG- and CCUG- containing transcripts accumulate in nuclear foci altering the function of the specific alternative splicing regulators, CUGBP1 and MBNL1, which are necessary for the physiological processing of mRNAs. These alterations lead to aberrant alternative splicing of different genes that explain different DM features (Taneja et al., 1995; Philips et al., 1998; Michalowski et al., 1999; Timchenko et al., 2001; Mankodi et al., 2001; Ranum and Day, 2004; Day and Ranum, 2005). However, the downstream pathways by which these RNA binding proteins cause skeletal muscle alteration such as the presence of very atrophic fibres, variability of myofibre size, ring fibres, and an increase in the number of fibres with central nuclei are not well understood (Meola 2000; Day and Ranum, 2005). For these reasons the general aim of this project was to understand the molecular mechanisms behind DM skeletal muscle alterations, focusing in particular on muscle atrophy.

In particular, we decided to study the molecular basis of skeletal muscle atrophy through the analysis of three different mechanisms potentially involved in this feature of myotonic dystrophy:

- The role of CUGBP1 and ZNF9/CNBP. Indeed, even if current evidence suggests that mutant CUG and CCUG repeats are responsible for muscle atrophy in DM1 and DM2, additional studies are needed to determine the contribution of the splicing regulators MBNL1 and CUGBP1 to the development of skeletal muscle alterations. Moreover, in DM2 patients the role of *ZNF9/CNBP* expression is still controversial and requires additional investigation since some DM2 patients show reduced protein levels but others do not (Botta et al., 2006; Margolis et al., 2006; Huichalaf et al., 2009; Pelletier et al., 2009; Raheem et al., 2010).
- The role of satellite cells. Indeed, it has been demonstrated that satellite cells isolated from DM1 patients reached the proliferative senescence when their mean telomere lengths were longer than those observed in passage-matched control cells indicating that DM1 cells reach proliferative arrest prematurely, potentially leading to skeletal muscle atrophy (Thornell et al., 2009). However, even if a recent study have shown that DM2 myoblasts exhibit senescence related features when cultured at early passages, additional studies are needed to elucidate if DM2 satellite cells activity is impaired as reported for DM1 possibly leading to

skeletal muscle atrophy.

- The role of insulin/IGF1 pathway. Indeed, it is known that this pathway promotes the synthesis and inhibits the degradation of muscle specific proteins and muscle atrophy can occur due to a misbalance between protein synthesis and protein degradation. Even if preliminary studies have shown that DM2 muscle cells show an alteration in proteins degradation system, other studies are necessary to better define the molecular alterations of the insulin/IGF1 pathway in both DM1 and DM2 patients (Rusconi et al., 2010).

The results will potentially lead to the identification of novel biomarkers that could be target for therapeutic intervention. Developing therapies for the prevention and treatment of muscle atrophy process will enhance the quality of life of patients who suffer from myotonic dystrophy and it would be beneficial to society as a whole since it would lead to a reduction in economic and productivity burdens associated with skeletal muscle damage.

4. Main Results

This project has been performed both *ex vivo* on biceps brachii human biopsies from control and DM patients and *in vitro* on myoblasts and myotubes cultures obtained from human control and DM biopsies.

In the first part of my PhD we have performed different studies to better define the molecular pathogenesis of DM. Indeed, even if it's known that the common key feature of DM pathogenesis is the nuclear accumulation of mutant RNA that causes the alteration in the activity of two RNA binding proteins, MBNL1 and CUGBP1, DM1 and DM2 show disease-specific features that make them separate diseases suggesting that other cellular and molecular pathways may be involved. In this background, we have analyzed the histopathological and biomolecular features of skeletal muscle biopsies from a cohort of DM1 and DM2 patients in relation to different phenotypes. On the basis of clinical phenotype, DM1 cohort has been divided in three phenotypes (5 patients with mild phenotype E1, 10 patients with classic phenotype E2 and 3 adult patients with Congenital Myotonic Dystrophy phenotype CDM). The DM2 cohort included 5 patients with a paucisymptomatic (PS) phenotype, 5 patients with Proximal Myotonic Dystrophy (PDM) phenotype and 10 patients with Proximal Myotonic Myopathy (PROMM). The histopathological analysis of muscle sections has shown that there is a positive correlation between the skeletal muscle impairment and the clinical phenotype. Indeed, the most severe histopathological alterations such as an increase in the atrophy and hypertrophy factor, the presence of nuclear clumps and an increase in central nucleation, were present in muscles from patients presenting the most severe clinical phenotype (DM1-E2, DM1-CDM, DM2-PDM, DM2-PROMM). On the contrary, as in control muscles, no histopathological changes were observed in muscles from both DM1-E1 and DM2-PS. In order to resolve the controversial results on CUGBP1 protein expression in DM2 muscle, we have examined the levels of this proteins in biceps brachii muscle samples. Our results indicated that an increase of CUGBP1 protein level is present in DM muscles as compared to controls even if not statistically significant due to the high interindividual variability observed in all groups. However, the increase of protein expression appears to be higher in DM1 than in DM2. Moreover, in DM1 the increase was evident only in DM1-E2 patients, while DM1-CDM and DM1-E1 showed similar levels to those observed in control muscles. On the contrary in DM2 only a slight increase of CUGBP1 expression was observable in DM2-PDM and DM2-PROMM but not in DM2-PS. Moreover the CUGBP1 levels in DM2-PDM and DM2-PROMM muscles appear to be lower than those observed in DM1-E2 muscles. Since it has been reported that the increase of CUGBP1 steady state protein level in DM1 cultured cells or animal models is related to protein hyperphosphorylation, we tested if the increase of CUGBP1 expression observed in our DM cohort is related to an increase of its phosphorylation.

Western blot analysis of the expression of CUGBP1 phosphorilated in S28 and 2D-GE analysis have shown that an hyperphosphorylation of CUGBP1 was present only in DM1-E2 patients which also showed the highest levels of CUGBP1 expression. In all the other groups, CUGBP1-p-S28 levels were similar to those observed in controls. The biomolecular analysis of alternative splicing alteration of IR, CLCN1, SERCA1, MBNL1 and CAPZB genes have shown that the frequency of abnormal isoforms are significantly increased in every DM1 and DM2 patient as compared to controls. Moreover, when considering single phenotypes, DM1-E1 and DM2-PS muscles showed a lower frequency of abnormal isoforms than those observed in the other DM1 and DM2 categories, confirming that a positive correlation between splicing alteration and clinical phenotype seems to be present. On the basis of the observed CUGBP1 increased protein levels in DM1 muscle, particular attention should be given to the expression of the CAPZB gene, which encodes for the F actin capping protein beta subunit. Indeed CAPZB splicing is dependent only on CUGBP1 and RT-PCR analysis showed that DM1-E2 patients exhibit the highest levels of its fetal isoform. Since it's known that elevated concentration of intracellular Ca^{2+} can lead to muscle degeneration and muscle atrophy, we have analyzed if there is a correlation between histopathological alterations in DM muscles and the expression of pathological isoform of SERCA1 which is one of the main regulators of intracellular Ca^{2+} homeostasis in skeletal muscle cells (Jacobs et al., 1990). The results indicated that there is a significant correlation between SERCA1 splicing alteration and the atrophy factor in DM1 but not in DM2 muscle. The results indicated that the splicing and muscle pathological alterations observed are related to the clinical DM1 and DM2 phenotype and that CUGBP1 seems to play a role only in DM1, confirming that the molecular pathomechanism of DM is more complex than the one actually suggested. In particular the reasons behind skeletal muscle histopathological alterations are still unclear. For these reasons, to better understand the molecular mechanisms underlying DM2 pathology, we decided to study the progression of the muscular involvement in relation to the evolution of skeletal muscle histopathology and biomolecular findings in DM2 patients. Indeed, we have analyzed at histopathological and biomolecular level the progression of the disease over time in 5 different DM2 patients. Our results indicated that a clear worsening in muscle histopathology over time was evident in every patient analyzed, where an increase in the percentage of type 2 fibres presenting central nucleation, in the number of fast positive nuclear clumps and in fibres atrophy were evident. On the contrary, no differences or only a slight increase of alteration in alternative splicing of some mRNAs (IR, CLCN1, SERCA1, MBNL1) was observable between the two biopsies. Moreover, as mentioned above, since it's known that elevated concentration of intracellular Ca^{2+} can lead to muscle degeneration and muscle atrophy, particularly attention should be given to the analysis of SERCA1 alternative splicing. This mRNA was found to

be more altered in the second biopsy only in 3 patients but with no correlation with the worsening in histopathological phenotype. Finally, since it is known that an alteration in ZNF9/CNBP protein expression can lead to a reduction in the rate of global protein synthesis, potentially explaining muscle atrophy in DM2, we decided to analyze CNBP protein levels in the two successive DM2 biopsies. Our results indicated that this protein appear to be reduced in DM2 muscles compared to control samples but again no differences were found between the two biopsies. All these data suggest that the molecular mechanisms that drive to skeletal muscle histopathological alterations and in particular to skeletal muscle atrophy are still unclear and that these features cannot be explained only by spliceopathy.

For all these reasons we decided to analyze DM satellite cells activity *in vitro*. Satellite cells are the muscle precursor cells that provide the potential for both pre and post-natal growth of skeletal muscles and for its regeneration following injury (Bischoff et al., 1994; Relaix et al., 2012). Since it has been observed that human DM1 myoblasts undergo senescence earlier than control cells, we decided to analyze if also DM2 satellite cells derived myoblasts exhibit a premature senescence as reported for DM1 (Thornell et al., 2009). The *in vitro* proliferative capacity of myoblasts obtained from skeletal muscle biopsies of 3 DM1 and 4 DM2 patients was compared to that of myoblasts obtained from 4 age matched unaffected individuals used as controls. Our results indicated that, even if an interindividual variability was evident in both DM1 and DM2 group, the average proliferative lifespan of the DM1 and DM2 myoblasts was reduced as compared to that observed in control cells. Since it is known that replicative senescence may be caused by progressive telomere shortening at each cellular division or by additional pathways such as the p16 stress pathway, we decided to anlalyse p16 expression in DM and control myoblasts at early and late stages of proliferation and telomeres shortening during *in vitro* aging. Interestingly our results indicated that p16 was overexpressed only in DM1 cells at both early and late stages of proliferation compared to controls, while no differences were found in DM2 and control myoblasts at both stages analyzed. On the contrary, telomere loss analysis have shown that only myoblasts isolated from DM1 patients stopped growing prematurely with telomeres longer than controls. Indeed, DM2 myoblasts stopped dividing with a median telomere length lower than controls and DM1 samples and had a clear decrease in the amount of telomeric DNA at every cell passage compared to controls. Finally, the analysis of myoblasts differentiative capability have shown that senescent cells from both control and DM patients were still able to form myotubes, however they appeared to be significantly smaller than those formed by the young cells, confirming that premature proliferative growth arrest of DM satellite cells might contribute to skeletal muscle atrophy in these patients.

Finally, to better understand the mechanisms behind skeletal muscle atrophy, we decided to

analyze another pathway involved in the regulation of skeletal muscle atrophy and hypertrophy which is the insulin/IGF1 pathway. Indeed DMs are characterized by metabolic dysfunctions such as insulin resistance, hyperinsulinemia, hypertriglyceridemia, increased fat mass, and a fourfold higher risk of developing Diabetes mellitus type 2 (T2DM) (Meola 2000). However in literature there are few studies aimed to clearly define the mechanisms underlying insulin resistance in DM. Savkur et al. (2001) suggested that splicing alteration of insulin receptor (IR) may play a role in peripheral insulin resistance. This splicing alteration leads to a higher expression of the fetal isoform A (IR-A, lacking exon 11) than the isoform B (Savkur et al., 2001). Moreover, the expression of IR in DM patients is still controversial since both normal and diminished insulin receptor (IR) RNA and protein levels have been reported (Savkur et al., 2001; Moxley et al., 1981; Morrone et al., 1997; Furling et al., 1999). Thus it cannot be excluded that post receptor signalling abnormalities could also contribute to the insulin resistance observed in DM patients and that insulin response defects might play a key role in the metabolic manifestations of DM, potentially leading to type II diabetes and abnormal muscle protein metabolism (Moxley et al., 1984, 1986). In this background, we decided to analyze the molecular mechanisms that induce insulin resistance in myotubes at five days of differentiation (T5) derived from myoblasts isolated from muscle biopsies of 3 DM1, 3 DM2 and 3 healthy subjects. Alternative splicing analysis of the insulin receptor (IR) have shown that at five days of differentiation (T5) both DM and control myotubes express more fetal isoform IR-A than the adult one (IR-B), confirming what was previously reported by Cardani et al. (2009). However, DM1 and DM2 muscle cells exhibited a lower glucose uptake after 10 nM insulin stimulation. Moreover, also the activation of several proteins involved in the insulin pathway (p70S6K, AKT, GSK3β) appeared to be lower in DM myotubes than in controls and this alteration seems to be more evident in DM2 muscle cells. It is known that the binding of insulin to its receptor activates a complex pathway culminating in the translocation of the glucose transporter GLUT4 into the plasma membrane. Since the cytoskeleton provides a platform for intracellular transport, we decided to investigate if actin and microtubule organization is impaired in DM skeletal muscle cells. Fluorescent analysis of actin remodelling in response to insulin stimulation did not show any difference between control and DM myotubes. However, microtubule nucleation analyzed by γ-tubulin staining has shown that DM muscle cells exhibit a defective microtubule reorganization after insulin stimulation. This alteration was associated with an increased activation of ERK1/2 and of GSK3β. These data indicate that post receptor signalling abnormalities might contribute to DM insulin resistance regardless the alteration of IR splicing.

5. Conclusions and Future Prospects

Myotonic dystrophy (DM) is an autosomal dominant multisystemic disorder characterized by a variety of multisystemic features including myotonia, muscular dystrophy, cerebral involvement, cardiac dysfunctions, cataracts and insulin-resistance (Meola and Moxley, 2004). However DM1 and DM2 present a number of very dissimilar features making them clearly separate diseases. For these reasons in the first part of my PhD we have performed different studies to analyze the histopathological and biomolecular features of skeletal muscle biopsies from a cohort of DM1 and DM2 patients in relation to different phenotypes. The results of our studies seem indicate that the splicing and muscle pathological alterations observed are related to the clinical phenotype. Indeed the most severe phenotype of both DM1 and DM2 showed the characteristic myopathic features of these diseases, while the less affected patients presented none or minimal muscle histopathological alterations. Moreover, as expected, alteration of alternative splicing of IR, CLCN1, MBNL1, SERCA1 and CAPZB genes was evident in both DM1 and DM2 muscle biopsies despite the clinical phenotype. To better define the molecular pathways which may be involved in disease-specific manifestations, we have analyzed the role of CUGBP1. Indeed, while it is clear that MBNL1 is depleted from nucleoplasm through recruitment into ribonuclear inclusions both in DM1 and DM2 even when clinical symptoms and muscle alterations are very mild, CUGBP1 overexpression has been clearly demonstrated in DM1 but not in DM2 muscle biopsies (Mahadevan et al., 2006; Jiang et al., 2004; Mankodi et al., 2005; Gates et al., 2011; Cardani et al., 2012). Our western blotting analysis of CUGBP1 protein expression confirms that CUGBP1 is overexpressed and more phosphorylated in DM1 muscle biopsies. However this increase was evident only in DM1-E2 while CUGBP1 protein levels in DM-E1 and DM1–CDM appeared to be similar to those observed in healthy controls. It has been suggested that in DM1 CUGBP1 may be responsible for muscle wasting since the transgenic mice with skeletal muscle-specific expression of CUGBP1 reproduces the dystrophic muscle histology characteristic of DM1, while MBNL1 knockout mice do not exhibit severe muscle wasting (Kanadia et al., 2003; Ward et al., 2010). In our work we have found a clear correlation between CUGBP1 expression and the atrophy factors found in DM1 muscles. However, when considering the different DM1 clinical phenotypes, DM1-E2 and DM1-CDM showed the higher values of atrophy factor and the most severe muscle histopathological alterations nevertheless CUGBP1 was overexpressed only in DM1-E2 muscles. Contrary to DM1, in DM2 muscle biopsies only a slight increase of the CUGBP1 protein levels was observed in DM2-PDM and DM2-PROMM but not in DM2-PS. However this increase was not related to an increase of protein phosphorylation. In addition our data on DM2 muscle seem to suggest that perturbation of CUGBP1 amount are not required to produce histopathological or splicing

regulation defects in DM2. Thus, since sequestration of MBNL1 evidently has a central role in splicing misregulation in both types of DM, it appears likely that in DM1 CUGBP1 overexpression might be an additional pathogenic mechanism not shared by DM2. These results confirm that the molecular pathomechanism of DM is more complex than the one actually suggested and in particular that the reasons behind skeletal muscle histopathological alterations are still unclear. All these results were confirmed by the analysis of the progression of the muscular involvement in DM2 patients. The analysis of two successive biopsies of five patients indicated that muscle morphological alterations evolve more rapidly over time than the molecular changes. Indeed, even if muscle degeneration appears to be more pronounced after a 10-year interval between the two successive biopsies, it was already evident after 2 years. This degenerative process may explain the worsening of muscle symptoms like weakness and wasting. Interestingly, this worsening of muscle symptoms in DM2 patients may be caused by the progressive enlargement of CCTG and by the consequent increase of the sequestration of MBNL1 that we observed in the second biopsy of every DM2 patient analyzed. Moreover, it has been suggested that a reduction of CNBP levels may play a role in DM2 pathology and might explain phenotypic differences between DM1 and DM2 (Chen et al., 2007; Huichalaf et al., 2009; Pelletier et al., 2009; Raheem et al., 2010). In this work we have observed a reduction of CNBP both at the mRNA and protein level in DM2 muscle, however this reduction was not related to a worsening of muscle histology. Finally, the analysis of alternative splicing of IR, SERCA1, CLCN1and MBNL1 genes in two successive muscle biopsies did not evidence a worsening of alternative splicing alterations even after a 10-year interval between the two biopsies. Moreover, since it's known that elevated concentration of intracellular Ca^{2+} can lead to muscle degeneration and muscle atrophy, particularly attention should be given to the analysis of SERCA1 alternative splicing. This mRNA was found to be more altered in the second biopsy only in 3 patients but with no correlation with the worsening in histopathological phenotype. All these data suggest that the molecular mechanisms that drive to skeletal muscle histopathological alterations and in particular to skeletal muscle atrophy are still unclear and that these features cannot be explained only by spliceopathy.

Since many symptoms of adult form of DM1 and DM2, such as muscle weakness and wasting, cataracts, and cardiac arrhythmias, are reminiscent to normal aging, we decided to analyze if also skeletal muscle atrophy can be linked to a premature aging of skeletal muscle. Supporting this thesis, recent data on dystrophic skeletal muscle myonuclei have demonstrated alterations of mRNA pathways similar to those observed during aging (Moss and Leblond, 1971; Malatesta et al., 2009; 2011; 2013). Moreover several studies have shown that in age-related myopathies, such as sarcopenia and myotonic dystrophy, the progressive muscle weakness and atrophy are characterized

by impaired muscle regeneration due to satellite cells premature senescence that limits their proliferative potential (Harper 2001; Bigot et al., 2009; Machida et al., 2006; Verdijk et al., 2007). Thornell et al. (2009) have shown that in DM1 muscles the number of satellite cells is increased compared to muscles from non-affected individuals; however, DM1 cells do not seem to be able to counteract the progressive muscle atrophy due to a reduced proliferative capacity triggered by a mechanism of premature growth arrest. To investigate if these alterations are evident also in DM2 patients, we have analyzed if cultured DM2 myoblasts differ from myoblasts of age-matched DM1 and normal individuals in terms of cell proliferation, morphology, differentiation and senescence during *in vitro* aging and if alterations in their proliferation potential and differentiation capabilities might contribute to some of the clinical and histopathological features observed in DM2 muscles. Our results seem indicate that DM1 and DM2 myoblasts are characterized by a premature proliferative growth arrest compared to healthy myoblasts suggesting that the *in vivo* regenerative capacity of DM satellite cells might be constitutively impaired. Interestingly, while the p16 pathway appeared to be responsible for the premature growth arrest in DM1, our results suggested that this mechanism was not responsible for the proliferative arrest observed in DM2 myoblasts (Bigot et al., 2009). On the contrary, DM2 myoblasts stopped dividing with telomeres shorter than controls suggesting that the signaling involved in premature senescence depend on a telomere-driven pathway and indicating that CCTG expansion might interfere with the telomere homeostasis in DM2 cells. However further analysis are necessary to clarify the mechanism causing an accelerated telomere shortening. Critically short telomeres trigger loss of cell viability in tissues, which has been related to alteration of tissue function and loss of regeneration tissue capabilities (Decary et al., 1997; 2000). This study has shown that CTG and CCTG expansions trigger *in vitro* a mechanism of myoblast premature senescence through two different pathways, which could explain the different histological alterations observed between DM1 and DM2 skeletal muscle. Moreover, as previously reported, our results indicated that replicative senescence deregulates the myogenic programme resulting in impaired myogenesis (Mathieu et al., 2001; Lorenzon et al., 2004; Bigot et al., 2009). Indeed, even though the senescent myoblasts are still able to fuse, a significant reduction in the fusion index has been observed when compared with young cells. Moreover, the reduction in fusion index appear to be more evident in senescent myoblasts obtained from DM1 patients indicating that myoblasts deficiency could be responsible of the more severe muscle histopathology observed in DM1 compared to DM2. Thus, the histopathological defects observed in DM muscle such as fibre atrophy and nuclear clumps, could be due at least in part to the inability of premature aged myoblasts to generate myotubes able to produce mature skeletal muscle fibers or to fuse with existing fibers and prevent them from atrophy.

Finally, to better understand the mechanisms behind skeletal muscle atrophy, we decided to analyze another pathway involved in the regulation of skeletal muscle atrophy and hypertrophy which is the insulin/IGF1 pathway. Indeed, when insulin binds its receptor, the activated IR tyrosine kinase phosphorylates several intracellular substrates, starting a complex cascade of biochemical signals that mediate the metabolic or mitogenic effects by the activation of PI3K or Ras pathway (Belfiore et al., 2009). The insulin PI3K/Akt pathway shares most of its components with the IGF1 (insulin-like growth factor 1) pathway intersecting at various levels. For example, insulin can also bind the IGF1 receptor and IGF1 can bind to the insulin receptor; furthermore, hybrids between the IGF1 and insulin receptors are present in skeletal muscle. It is known that insulin and IGF play an important role in skeletal muscle growth, development, differentiation and regeneration. They promote the synthesis and inhibit the degradation of muscle specific proteins through two main downstream effectors: mTOR, which controls protein synthesis, and FoxO, which controls protein degradation via the proteasomal and autophagic/lysosomal systems. Moreover, in absence of insulin stimulus, a loss in muscle mass and strength can be observed (Harridge 2003; Schiaffino et al., 2011; 2013). Progressive muscle wasting and weakness are very characteristic features of both DM1 and DM2 but, unlike other forms of muscular dystrophy, they do not result from fiber degeneration accompanied by muscle fibrosis. Indeed, several studies have shown that muscle atrophy in myotonic dystrophy reflects a selective decrease in muscle protein synthesis suggesting that muscle wasting could be a result from a defect in muscle anabolism (Halliday et al., 1985; Griggs et al., 1990). Moreover, DMs are characterized by metabolic dysfunctions such as insulin resistance, hyperinsulinemia, hypertriglyceridemia, increased fat mass, and a fourfold higher risk of developing Diabetes mellitus type 2 (T2DM) (Meola 2000). However, in literature there are few studies aimed to clearly define the mechanisms underlying insulin resistance in DM. Savkur et al. (2001) suggested that splicing alteration of insulin receptor (IR) may play a role in peripheral insulin resistance. This splicing alteration leads to a higher expression of the fetal isoform A (IR-A, lacking exon 11) than the isoform B (Savkur et al., 2001). IR-B differs from IR-A by the inclusion of exon 11 that leads to a higher insulin signalling capability. However, it cannot be excluded that post receptor signalling abnormalities could also contribute to the insulin resistance observed in DM patients and that insulin response defects might play a key role in the metabolic manifestations of DM, potentially leading to type II diabetes and abnormal muscle protein metabolism (Moxley et al., 1984; 1986). In this background, we decided to analyze the molecular mechanisms that induce insulin resistance in myotubes at five days of differentiation (T5). Alternative splicing analysis of the insulin receptor (IR) have shown that at five days of differentiation (T5) both DM and control myotubes express more fetal isoform IR-A than the adult one (IR-B). However, DM1 and DM2

muscle cells exhibited a lower glucose uptake after 10 nM insulin stimulation. This result is consistent with what was previously reported by Furling et al. (2009): these authors have shown that in CDM myotubes a dose of 10 nM insulin produced no stimulatory effect on glucose uptake and on protein synthesis. Moreover, also the activation of several proteins involved in the insulin pathway (p70S6K, AKT, GSK3β) appeared to be lower in DM myotubes than in controls and this alteration seems to be more evident in DM2 muscle cells. All these data indicate that post receptor signalling abnormalities might contribute to DM insulin resistance regardless the alteration of IR splicing. However further investigations will be necessary to understand whether these alterations may contribute to the histopathological changes observable in skeletal muscle. The results will lead to the identification of novel therapeutic approaches to prevent these features of the disease. Indeed, metformin is now considered as the first line drug for insulin resistance diseases, including DM, and it increases glucose uptake in muscle through an insulin-indipendent pathway (Kouki et al., 2005). In the recent years some important component of many foods like Resveratrol, Betaine and Carnitine have found to be insulin mimetic compounds since they activate insulin/IGF1 signalling pathway leading to hypertrophic effects of C2C12 murine muscle cells (Montesano et al., 2013; 2015; Senesi et al., 2013). Moreover Resveratrol has been shown to act on skeletal muscle metabolism and function and recently its influence on alternative splicing of pre-mRNA was studied on DM1 fibroblasts where it enhanced the inclusion of exon 11 of the *IR* gene, providing a justification of resveratrol as a leading compound to improve glucose tolerance in DM1 (Takarada et al., 2015). For these reasons we intend to study the effects of resveratrol, betaine and carnitine on insulin resistance and skeletal muscle atrophy in DM patients as alternative drugs to metformin since they are insulin mimetic natural compounds that might have lower side effects.

In order to investigate the molecular mechanisms that induce insulin resistance in skeletal muscle in both DM1 and DM2 patients, we decided to analyze cytoskeleton organization in DM1 and DM2 muscle cells. Indeed, it is known that the binding of insulin to its receptor activates a complex pathway culminating in the translocation of the glucose transporter GLUT4 into the plasma membrane and the cytoskeleton provides a scaffold for intracellular transport such as the movement of vesicles and organelles. The importance of identifying the molecular mechanisms by which cytoskeletal elements contribute to GLUT4 translocation is underscored by recent studies that highlight defects in actin dynamics in conditions of insulin resistance (JeBailey et al., 2007; Zaid et al, 2008). Our results indicated that in control myotubes there is an insulin-dependent reorganization of microtubules that is associated with a translocation of GLUT4 and in the uptake of glucose. These results are consistent with what other authors have previously reported. Indeed, some studies have shown that microtubule depolymerizing agents such as nocodazole attenuate the

insulin-stimulated plasma membrane fusion of GLUT4 (Liu et al., 2013; Fletcher et al., 2000). Interestingly, γ-tubulin staining has shown that DM muscle cells exhibit a defective microtubule reorganization after insulin stimulation. Moreover, DM myotubes exhibited an increase in basal microtubule nucleation as compared to controls. These results seem indicate that DM muscle cells have a global alteration in microtubule nucleation and stabilization. This alteration was consistent with the observed increased activation of ERK1/2 and GSK3β. Indeed, polymerization and stability of microtubules is regulated by a number of microtubule associated proteins (MAPs) and moreover ERK and GSK3β have been shown to phosphorylate and regulate the binding activity to microtubules of these proteins. ERK1/2 (extracellular signal-regulated kinases) belong to the family of MAPK (mitogen-activated protein Kinases) and are strongly activated by growth factors, cytokines, osmotic stress, and microtubule disorganization (Lewis et al., 1998). Moreover, once activated, they phosphorylate numerous substrates in all cellular compartments, including cytoskeletal proteins (Chen et al., 2001). GSK3β is a constitutively active protein kinase whose activity is inhibited by phosphorylation upon insulin stimulation. Interestingly, type 2 Diabetes (T2D) is strongly associated with a decrease in insulin-stimulated glycogen synthesis along with increased GSK3β protein levels in the muscle (Bogardus et al., 1984; Shulman et al., 1990; Kelley et al., 1996; Cline et al., 1999). These data indicate that microtubule abnormalities might contribute to insulin resistance observed in DM myotubes. However further analysis are necessary to understand if an alteration in other cytoskeletal proteins may be involved in the molecular mechanism that induce insulin resistance in myotonic dystrophy. Indeed several studies have shown that in DM1 and DM2 patients several proteins involved in the cytoskeleton and in vesicle transport undergo to an aberrant alternative splicing, supporting the hypothesis that GLUT4 trafficking, docking and fusion could be altered in DM muscle cells leading to insulin resistance (Nakamori et al., 2013; Perfetti et al., 2014). Moreover, further investigations will be necessary to identify if microtubule instability might be related to other clinical features of this disease.

In conclusion, all these data suggest that the molecular mechanisms behind skeletal muscle atrophy in DM patients is more complicated than the one suggested and moreover this feature cannot be explained only by spliceopathy. Moreover, even if further investigations are necessary to understand how CTG and CCTG expansions lead to an alteration in satellite cells activity and in the regulation of the insulin/IGF1 pathway, our analysis have shown that the pathogenetic mechanisms in DM1 and DM2 present a number of very dissimilar features that make them clearly separate diseases.

6. References

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Part II

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Overexpression of CUGBP1 in Skeletal Muscle from Adult Classic Myotonic Dystrophy Type 1 but Not from Myotonic Dystrophy Type 2

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Abstract

Myotonic dystrophy type 1 (DM1) and type 2 (DM2) are progressive multisystemic disorders caused by similar mutations at two different genetic loci. The common key feature of DM pathogenesis is nuclear accumulation of mutant RNA which causes aberrant alternative splicing of specific pre-mRNAs by altering the functions of two RNA binding proteins, MBNL1 and CUGBP1. However, DM1 and DM2 show disease-specific features that make them clearly separate diseases suggesting that other cellular and molecular pathways may be involved. In this study we have analysed the histopathological, and biomolecular features of skeletal muscle biopsies from DM1 and DM2 patients in relation to presenting phenotypes to better define the molecular pathogenesis. Particularly, the expression of CUGBP1 protein has been examined to clarify if this factor may act as modifier of disease-specific manifestations in DM. The results indicate that the splicing and muscle pathological alterations observed are related to the clinical phenotype both in DM1 and in DM2 and that CUGBP1 seems to play a role in classic DM1 but not in DM2. In conclusion, our results indicate that multisystemic disease spectrum of DM pathologies may not be explained only by spliceopathy thus confirming that the molecular pathomechanism of DM is more complex than that actually suggested.

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Introduction

Myotonic dystrophy (DM) is the most common adult onset muscular dystrophy affecting mainly skeletal muscle, heart, and the central nervous system [1]. Two DM loci are associated with two types of the disease. DM type 1 (DM1) is caused by the expansion of an unstable CTG trinucleotide repeat in the 3′ untranslated region of the DM protein kinase (*DMPK*) gene [2,3]. The DM type 2 (DM2) mutation consists in the expansion of an unstable CCTG tetranucleotide within the first intron of the CCHC-type zinc finger, nucleic acid-binding protein (*CNBP*) gene (previously named *Zinc Finger Protein 9, ZNF9* gene) [4]. Both DM1 and DM2 are progressive multisystemic disorders characterized by muscle weakness, myotonia, cataracts, cardiac conduction defects, cerebral involvement and endocrinological disturbances such as increased insulin resistance and male hypogonadism. Experimental evidence supports an RNA gain-of-function mechanism of expanded transcripts in both DM1 and DM2 in which repeat containing transcripts from the expanded allele accumulate in nuclei as foci and alter the functions of RNA binding proteins involved in regulating alternative splicing and mRNA translation [5,6]. The alteration of pre-mRNA processing strengthens the hypothesis of a spliceopathy which leads to inappropriate expression of embryonic splicing isoforms in adult tissues thus explaining, at least in part, the multisystemic aspect of the disease [7]. Expanded CUG/CCUG repeats mediate their effects on alternative splicing regulation through at least two RNA binding proteins: muscleblind like 1 (MBNL1) and CUGBP/Elav-like family member 1 (CELF1/CUGBP1) [8]. MBNL1 preferentially recognizes CUG or CCUG repeats when they are pathologically expanded [9,10] and is sequestered by ribonuclear foci in DM1 and DM2 cells [9,11,12] resulting in a loss of MBNL1 activity. In contrast, CUGBP1 does not colocalize with ribonuclear foci in DM1 cells [9,13,14], however this protein may have a role in the pathogenesis of splicing abnormalities because it is overexpressed in DM1 myoblasts, skeletal muscle and heart tissues [15–17].

Although DM1 and DM2 have similar clinical and genetic characteristics, they also present a number of very dissimilar features. DM1 is characterized by the phenomenon of anticipation, by which the disease has an earlier onset and more severe course in subsequent generations. Thus the clinical spectrum of DM1 include four main categories, each presenting specific clinical features: the congenital form that presents the most severe phenotype characterized mainly by CNS involvement and mental

retardation, the childhood-onset form with school mating and psychological problems, the adult-onset ("classical" DM1) where the core features are facial weakness with ptosis, myotonia and distal muscle weakness, and the late-onset or oligosymptomatic patients where only limited features are found on clinical and paraclinical assessment. The DM1 mutation length predicts the clinical outcome to some extent: oligosymptomatic 50–100 repeats, classical DM1 100–1.000 repeats; congenital >1.000 repeats [18,19]. There is a relative correlation between the length of CTG repeat expansions and age of onset for DM1 patients with CTG <400, but correlation between repeat length and disease severity is poor for long repeats [1,20,21]. In DM2 there are no distinct clinical subgroups although initially, different phenotypes of DM2 with proximal muscle weakness were described: DM2/ Proximal Myotonic Myopathy (PROMM) and Proximal Myotonic Dystrophy (PDM) [22–25]. PDM patients show many features similar to those found in PROMM, including proximal muscle weakness, cataracts, and electrophysiologically detectable myotonia. Unlike PROMM patients, however, they do not report myalgias, symptomatic myotonia, or muscle stiffness. Instead they present traits not present in PROMM, such as pronounced dystrophic-atrophic changes in the proximal muscles and late-onset progressive deafness [24]. The most important discrepancy between DM1 and DM2 is absence of a congenital form in DM2 [26,27]. In DM2 the smallest reported mutation vary between 55–75 CCTG [4,28] and the largest expansions have been measured to be up about 11.000 repeats [4], however the size of CCTG repeat expansion in leukocyte DNA in DM2 seems to relate in large part to the age of the patient and not necessarily to the severity of symptoms or manifestations. Despite the CCUG expansions are longer than DM1 CUG expansions, DM2 shows a less severe phenotype. Clinical myotonia is usually milder in DM2 and histopathological features in DM1 and DM2 are also different. In DM2 a subpopulation of extremely atrophic type 2 fibers, including the nuclear clump fibers, are present [1,29,30].

Recent studies have indicated that cardinal features of DM1 can be reproduced in the absence of nuclear inclusions and that RNA foci formation and splicing defects are separable [31,32]. Moreover, DM1-associated splicing defects have been observed in mouse models of other muscular dystrophies indicating that spliceopathy is secondary to muscle damage [33]. However to date, literature has been focused on reinforcing the prevailing common model of DM pathogenesis based on the presence of mutant RNA foci in cell nuclei and spliceopathy. On the other hand, the existence of disease-specific features that make DM1 and DM2 clearly separate diseases and the existence of DM1 and DM2 distinct subtypes suggest that other cellular and molecular pathways are involved besides the shared pathogenetic model hypothesized. Moreover, the RNA gain of function toxicity has been better characterized in DM1 than in DM2 probably due to a greater availability of DM1 samples and mouse models. Importantly, the role of CUGBP1 in DM2 is particularly intriguing with contradictory results being reported. Indeed it appears that in DM1 a combined effect of decreased MBNL1 and increased CUGBP1 activity lead to misregulated alternative splicing and other changes of the muscle transcriptome [5,34]. Instead in DM2, splicing abnormalities are also associated with the sequestration of MBNL1 protein by expanded transcripts [5,12], however evidence that CUGBP1 upregulation also occurs in DM2 is conflicting [34–36]. Timchenko and colleagues reported an increase of CUGBP1 in DM2 cultured myoblasts and muscle biopsies analyzing cytoplasmic extracts [34]. Moreover they reported that expression of pure RNA CCUG repeats in normal human myoblasts, in C2C12 cells and in a DM2 mouse model also increased levels of CUGBP1 [34]. On the contrary, in two different reports, the analysis of total cellular extract from DM2 cultured myoblasts and from muscle biopsies of DM2 patients did not show differences in CUGBP1 levels [35,36]. Nevertheless, it should be noted that in these works no mention is made of either the number or the clinical features and muscle histopathology of the patients used. In DM2 patients the role of ZNF9/CNBP expression is still controversial and requires additional investigation since some DM2 patients show reduced protein levels but others do not [36,37–40]. In this study we have analysed the histopathological, biochemical and molecular features of skeletal muscle biopsies from DM1 and DM2 patients in relation to presenting phenotypes (mild-E1 *vs.* classic-E2 *vs.* CDM in DM1 and PROMM *vs.* PDM *vs.* paucisymptomatic in DM2). This is the first study where the expression of CUGBP1 protein has been examined in a large cohort of DM2 patients. Moreover, DM2 muscle biopsies have been characterized together with several DM1 and control samples. This work intends to clarify which factors may act as modifiers of disease-specific manifestations in DM beyond spliceopathy.

Materials and Methods

Patients and skeletal muscle samples

This study was authorized by the Institutional Ethics Committee (ASL MI2-Melegnano via VIII Giugno, Milan) and was conducted according to the principles expressed in the Declaration of Helsinki, the institutional regulation and Italian laws and guidelines. All blood samples and muscle biopsies were used for this study after receiving written informed consent from the patients. With regard to children participants, we have obtained written informed consent from their parents.

Human muscle biopsies from biceps brachii muscle were taken under sterile conditions from 18 DM1, 20 DM2 patients and from 8 age-matched subjects who underwent muscle biopsy and resulted negative. Muscle samples were trimmed of blood vessels, fat and connective tissues and then fresh-frozen in isopentane cooled in liquid nitrogen. The diagnosis of DM was based upon the clinical diagnostic criteria set by the International Consortium for Myotonic Dystrophy [41]. Fluorescence in situ hybridization was performed on DM2 muscle frozen sections using a $(CAGG)_5$ probe as previously reported by Cardani et al. [42] to verify the presence of ribonuclear inclusions.

Genetic analysis of CTG and CCTG expansions

For DM1 genotyping, 1 μg of genomic DNA of each patients extracted from peripheral blood leukocytes were by "*Myotonic Dystrophy SB kit*" (Experteam s.r.l, Venezia, Italy). Forward primer was labelled at the 5' end with fluorescent tag 6-FAM. PCR conditions were: one cycle of 1 min at 94°C; 28 cycles of 20 sec at 94°C and 7 min at 62°C; and finally 10 min at 72°C. The amplifications were performed by MyCycler instrument (BioRad). After the amplification 20 μl of each PCR products were run on 3.5% MetaPhore agarose gel at 100V and stained with ethidium bromide. Alleles with less than 100 repeats were analyzed by capillary electrophoresis on 3500 Genetic Analyzer (Applied Biosystems) using LIZ600 as size standard. The analysis of results was performed using GeneMapper v4.1 (Applied Biosystems). For alleles with more than 100 repeats Southern blot hybridization was performed using a non-radioactive Digoxigenin-based probe 5'DIG- labelled $[CTG]_{10,}$ and the [CTG] repeats size was determined comparing the bands pattern obtained by Southern Blot Analysis with two DNA Molecular Weight Markers VII and VIII, DIG-labelled (Roche Diagnostics). DM2 genotyping has

been performed on genomic DNA extracted from peripheral blood leukocytes by long-PCR analysis as described [37,43].

Muscle histopathology

Muscle tissue was fresh-frozen in isopentane cooled in liquid nitrogen. Histopathological analysis was performed on serial sections (8 μm) processed for routine histological or histochemical stainings. A standard myofibrillar ATPase staining protocol was used after preincubation at pH 4.3, 4.6, and 10.4 [44]. The most typical alterations, such as nuclear clump fibers (i.e. aggregates of myonuclei with a thin rim of cytoplasm), nuclear centralization and fiber size variability were evaluated on serial muscle sections.

Immunohistochemistry

Serial transverse muscle cryostat sections 6 μm thick were cut for immunohistochemical staining (IHC). Sections were air-dried and rehydrated in phosphate buffer pH 7.4 (PBS). Non-specific binding sites were blocked with normal goat serum (NGS; DAKO) at a dilution 1:20 in PBS containing 2% bovine serum albumin (BSA; Sigma-Aldrich) for 20 min at room temperature (RT). Mouse monoclonal primary antibodies against two different myosin heavy chain (MHC) isotypes were used at the following dilutions: MHCfast, 1:400 in PBS+2% BSA (Sigma-Aldrich); MHCslow, 1:400 in PBS+2% BSA (Sigma-Aldrich). Each antibody was applied for 1 h at RT. After washing in PBS 3 times for 5 min, sections were incubated with goat anti-mouse biotinylated secondary antibody diluted 1:300 in PBS+2% BSA for 1 h at RT. After PBS washing, sections were incubated with StreptABComplex (DAKO) for 30 min and then exposed to the 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen reaction solution for 10 min. Nuclei were counterstained with Mayer's hematoxylin. Quantitative evaluation of fiber diameter was made as described previously by Vihola et al. [29] on images taken with a slide scanner ScanScope CS (Aperio Technologies, Vista, CA, USA) using the slide scanner software ImageScope. The size of muscle fibers was assessed by measuring the "smallest fiber diameter." All data were elaborated using Microcal Origin (Microcal Software Inc., Northampton, MA, USA).

Western blot analysis

Whole cell extracts were obtained from fifteen-twenty consecutive muscle cryostat sections 10 μm thick homogenized in 60 μl of 50 mM TrisHCl with 5% SDS (pH 7.5). After incubating on ice for 15 min, samples were centrifuged at 5,700 *g* for 12 min at 4°C, and supernatant was collected and stored at −80°C. Pellets were resuspended in 50 mM TrisHCl with 5% SDS (pH 7.5) and stored at −80°C. Protein concentration in each sample was determined by using BCA Protein Assay (Bio-Rad Laboratories). An equal amount of protein was loaded per lane and electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gels, and then transferred to nitrocellulose Protran membranes (Schleicher & Shuell GmbH). After blocking non specific sites in TrisHCl buffer pH 7.5 (TBS) containing 5% BSA for 30 min at 42°C, membranes were incubated overnight at 4°C with rabbit polyclonal anti-CUGBP1-posphoS28 (Abnova; 0.5 μg/ml), with mouse monoclonal anti CUGBP1 (Santa Cruz; clone 3B; 1:1000), or with rabbit polyclonal anti-ZNF9/CNBP (1:1000) [45]. After several washes in TBS+0.2% Tween20 or TBS+0.3% Tween20, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) diluted 1:5000 or 1:10000 in TBS+5% BSA+ 0.2% Tween20 respectively. Membranes were washed and immune complexes were detected using the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). GAPDH (polyclonal antibody diluted 1:80000; Sigma–Aldrich) was used as internal control to verify and correct for loading error. Blots have been performed in triplicate.

Two-dimensional gel electrophoresis (2D-GE)

6 DM1 (3 DM1-E1, 3 DM1-E2), 6 DM2 (3 DM2-PDM, 3 DM2-PROMM) and 6 control samples have been analysed. Each sample containing 50 μg proteins was resuspended in a solution containing 7 M urea, 2 M thiourea, and 4% 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (CHAPS). Samples were used to rehydrate immobilized pH gradient (IPG) strips just before isoelectrofocusing. For the first-dimension electrophoresis, samples were applied to IPG strips (11 cm, pH 3–10 linear gradient; GE Healthcare). Strips were rehydrated at 20°C for 1 h without current and for 12 h at 30 V in a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1 mM dithiothreitol (DTT), and 1% IPG buffer 3–10 (GE Healthcare). Strips were focused at 20°C for a total of 70,000 V/h at a maximum of 8000 V using the Ettan IPGphor II system (GE Healthcare). The focused IPG strips were stored at −80°C. For the second dimension, IPG strips were equilibrated at room temperature for 15 min in a solution containing 6 M urea, 2% SDS, 30% glycerol, 50 mM Tris–HCl (pH 8.8), and 10 mg/ml DTT and then reequilibrated for 15 min in the same buffer containing 25 mg/ml iodoacetamide in place of DTT. The IPG strips were placed on top of a 12% polyacrylamide gel and proteins were separated at 25°C with a prerun step at 20 mA/gel for 1 h and a run step at 30 W/gel for 3.5 h. After run, gels were transferred to nitrocellulose Protran membranes (Schleicher & Shuell GmbH). CUGBP1 and GAPDH, used to normalize protein load on IPG strip, have been immunodetected as described above.

Study of alternative splicing

Frozen muscle samples were practiced for the extraction of total RNA using TRIzol reagent (Gibco BRL, Gaithersburg, MD) and 1 μg of RNA was reverse transcribed according to the cDNA protocol of the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Splicing pattern profile of the *IR*, *CLCN1*, *MBNL1*, *SERCA1* and *CAPZB* genes was carried out as described [46–48]. Total PCR products, obtained within the linear range of amplification, were electrophoresed on 2.5% agarose gel. Quantitative analysis of the amplified products was performed using SybrGreenII-stained gels (Perkin-Elmer Life Science, Massachusetts, USA) scanned on a fluorimager 595 (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band and the fraction of abnormally (or pathologically) spliced (AS) isoforms (AS-isoforms/total) were quantified by densitometry using ImageQuant software. Statistical methods were used to analyze the differences in the identified splice variants between DM1 and DM2 patients respect to controls. Control of the RT-PCR reaction was based on the expression level of the glucose phosphate isomerase housekeeping gene (GPI) and all amplifications have been carried out in triplicate using independent cDNA samples.

QRT-PCR expression analysis of the CLCN1, ZNF9/CNBP and CUGBP1 genes

Following RNA extraction and retro-transcription, cDNA of DM samples were also used to quantify the expression level of the *CLCN1*, *ZNF9/CNBP* and *CUGBP1* genes. The total expression of mentioned genes was evaluated using specific TaqMan gene expression assays: *CLCN1* [Hs00163961_m1], *ZNF9/CNBP* [Hs00231535_m1] and *CUGBP1* [Hs00198069_m1] (Applied Biosystems). The VIC-labelled β_2-microglobulin gene (*B2M*: GenBank accession #NM_004048) was used as housekeeping

Table 1. Clinical data on DM patients used in this study.

	Phenotype	Clinical features	Age at biopsy	MRC[a]	Diabetes	EKG[b] Abnormalities %
				tot	%	
Controls	Healthy subjects	No clinical signs	45.2±13.8	149.3±1.0	0%	0%
(n = 8)						
DM1 (n = 18)	Mild (n = 5)	Minimal clinical signs	41.2±17.5	149.6±1.7	0%	0%
	(E1: 50<CTG<150)	(MIRS[c] = 2)				
	Classic (n = 10)	Overt clinical symptoms	44.3±10.5	118.9±19.3	0%	60%
	(E2: 150<CTG<1000)	(MIRS = 3-4)				
	Congenital Myotonic Dystrophy (n = 3)	Symptoms at birth	22.7±10.7	114.7±4.2	0%	100%
	(CDM: CTG>1000)					
DM2 (n = 20)	Paucisymptomatic (n = 5)	Absence of muscular weakness	38.0±13.4	149.6±1.0	0%	0%
	(PS)					
	Proximal myotonic dystrophy (n = 5)	Severe atrophy	65.0±8.4	131.4±11.4	40%	0%
	(PDM)	No clinical myotonia				
	Proximal myotonic myopathy (n = 10)	Proximal muscle weakness	55.0±6.1	142.6±2.5	20%	20%
	(PROMM)	Myotonia				

[a]Medical Research Council, scale for muscle strength; scale (0–5 grade) on 15 muscles at both sides in the upper and lower limbs for a total of 150 maximum score.
[b]Electrocardiogram, included first-degree atrio-ventricular block, incomplete or complete bundle-branch block.
[c]Muscle Impairment Rating Scale, stage of the disease for DM1 patients [73].
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internal control gene, as described [49]. The simultaneous measurement of genes-FAM/B2M-VIC expression allows to normalize the amount of cDNA added per sample. Each PCR reaction was performed in triplicate using the TaqMan Universal PCR Master Mix and the ABI PRISM 7500 Fast System (Applied Biosystems). A comparative threshold cycle (Ct) was used to determine *CLCN1* and *ZNF9/CNBP* genes expression compared to a calibrator (median value of control subjects). Hence, steady-state mRNA levels were expressed a *n*-fold difference relative to the calibrator. For each sample, genes' Ct value was normalized using the formula ΔCt = Ct genes – Ct B2M. To determine relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct$ sample $- \Delta Ct$ calibrator. The value adopted to plot relative gene expression was calculated using the expression $2^{-\Delta\Delta Ct}$. The relative quantification of the *CUGBP1* mRNA steady-state level was calculated using the Pfaffl equation accordingly to Pfaffl et al [50].

Statistical analysis

Overall statistical significance has been calculated by using the Kruskal-Wallis test (non parametric ANOVA) and significant differences between groups have been determined using the Dunn's multiple comparisons post-test.

Results

Patients

On the basis of clinical phenotype, DM1 cohort has been divided in three subphenotypes: 5 DM1 patients with mild phenotype (E1), 10 DM1 patients with classic phenotype (E2) and 3 DM1 adult patients with Congenital Myotonic Dystrophy phenotype (CDM). The DM2 cohort included: 5 DM2 patients with a paucisymptomatic (PS) phenotype, 5 DM2 patients with Proximal Myotonic Dystrophy (PDM) phenotype (severe atrophy and myotonia only at EMG) and 10 DM2 patients with Proximal Myotonic Myopathy (PROMM) phenotype (proximal muscle weakness and myotonia). Data on DM patients used in this study are reported in Table 1.

Muscle histopathology

Analysis of muscle sections immunostained for MHC fast and slow myosin allows us to detect and measure fibers smaller than 5 μm, including all nuclear clump fibers which are recognizable by the presence of a thin rim of immunoreaction around the nuclei. The metahistograms based on the analysis of fiber diameters on immunostained muscle sections and the evaluation of atrophy (AF) and hypertrophy (HF) factors are reported in Figure 1. An increase of both type 1 and type 2 fiber AF is present in DM1-E2 and DM1–CDM (Figs. 1B, C). The AF increase is not present in DM1-E1 (Fig. 1A). Type 2 fiber atrophy is most evident in DM1-E2 which shows a bimodal size distribution histogram of type 2 fibers (Fig. 1B). Among DM2 muscles, DM2-PDM and DM2-PROMM show an increase of both AF and HF not evident in DM2-PS (Figs. 1D-F). In DM2-PROMM muscles, atrophy affects type 2 but not type 1 fibers whereas in DM2-PDM a slight increase of type 1 fiber AF is also present (Figs. 1E, F). Both DM2-PDM and DM2-PROMM exhibit a bimodal size distribution histogram of type 2 fibers. The most severe histopathological alterations are present in muscles from patients presenting the most severe clinical phenotype i.e. DM1-E2, DM1-CDM, DM2-PDM and DM2-PROMM. In DM1-E2 and DM1-CDM, nuclear clumps fibers and highly atrophic fibers express MHC fast myosin and a coexpression with MHC slow myosin is evident in most of them (Figs. 2C, D). In DM2-PDM and DM2-PROMM muscles, numerous nuclear clumps fibers expressing only MHC fast myosin

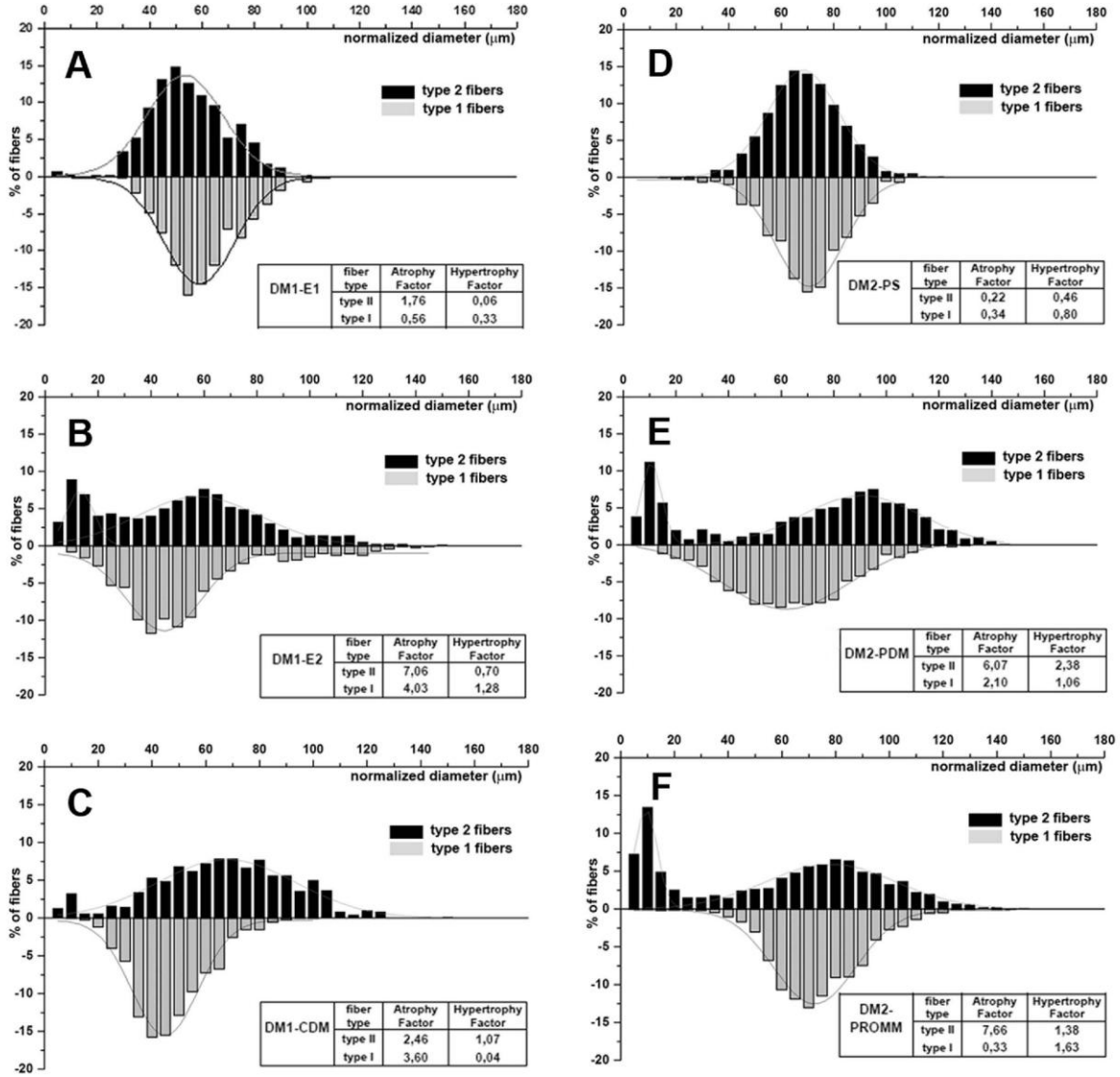

Figure 1. Metahistograms have been obtained from the analysis of muscle fiber diameters in DM1 patients (A-C) and in DM2 patients (D-F). The results are based on sections immunostained for MHC fast or slow myosin. Tables show the relative atrophy or hypertrophy factors in each subphenotype considered. Data relative to each DM1 and DM2 phenotypic groups have been obtained by pooling the findings of each patient: DM1-E1 (n = 3), DM1-E2 (n = 5), DM1-CDM (n = 3), DM2-PS (n = 4), DM2-PDM (n = 5) and DM2-PROMM (n = 5).
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are present (Figs. 2F, G). Central nucleation is always present and involved prevalently type 1 fibers in DM1 muscles and type 2 fibers in DM2 muscles. As in control muscles, no histopathological changes are instead observed in muscles from both DM1-E1 and DM2-PS where no nuclear clumps fibers or central nuclei are present (Figs. 2A, B, E).

CUGBP1 protein expression is more elevated in DM1 than in DM2 skeletal muscle

In order to resolve the controversial results on CUGBP1 protein expression in DM2 muscle, we have examined the protein levels of CUGBP1 in biceps brachii muscle samples from DM1, DM2 and control individuals by western blotting analysis (Fig. 3A). An increase of CUGBP1 protein level is present in DM muscles as compared to controls even if not statistically significant due to the high interindividual variability observed in all groups. However, the increase of protein expression appears to be higher in DM1 than in DM2 (Fig. 3B). When considering the 3 DM1 different phenotypes separately, the increase is evident only in DM1-E2 while in DM1-CDM and DM1-E1 muscles the protein levels appear to be equal to those observed in control muscles (Figs. 3C). A clear correlation between the AFs and the CUGBP1 expression levels has been observed in DM1 muscles ($p<0.01$, data not

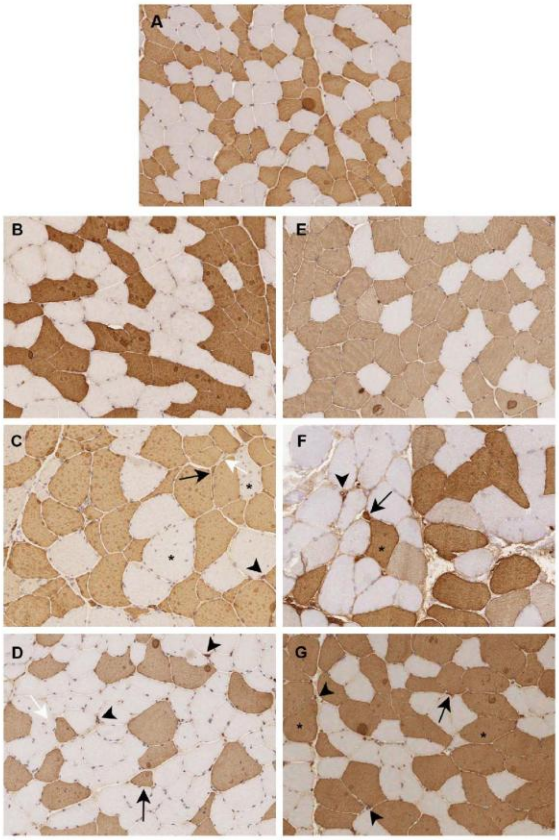

Figure 2. Fast myosin immunostaining of skeletal muscle transversal sections obtained from a healthy patient (A), DM1 patients (B-D) and DM2 patients (E-G). Type 2 fibers (fast positive fibers) are stained in brown. Muscle from DM1-E1 (B) and DM2-PS (E) patients show a normal histological muscle pattern similar to those observed in control muscle section (A). Muscle from DM1-E2 (C) and DM1-CDM (D) patients show a high fiber size variability with both type 1 (unstained fibers; white arrows) and type 2 (black arrows) atrophic fibers, fast positive nuclear clumps (arrowheads) and a preferential type 1 fiber central nucleation (asterisks). Muscle from DM2-PDM and DM2-PROMM patients also show high fiber size variability with very small type 2 fibers (black arrows), type 2 nuclear clumps (arrowheads) and a preferential type 2 fiber central nucleation (asterisks).
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shown). A slight increase in CUGBP1 protein expression is observable in DM2 muscles compared to controls and this increase is present in DM2-PDM and DM2-PROMM phenotypes but not in DM2-PS. However the CUGBP1 levels in DM2-PDM and DM2-PROMM muscles appear to be lower than those observed in DM1-E2 (fold increase 1.3 *vs* 1.6 compared to controls) (Fig. 3C). Nevertheless, it has been reported that the increase of CUGBP1 steady state protein level in DM1 cultured cells or animal models is related to protein hyperphosphorylation [51]. Several kinases phosphorylate CUGBP1 at different residues and multiple functions of the protein are regulated by phosphorylation at distinct sites. While the specific sites of phosphorylation by PKC have not yet been identified, it has been demonstrated that Akt phosphorylates CUGBP1 at serine-28 (S28) and cyclin D3/cdk4 at serine 302 (S-302) [39,51,52]. Since it has been reported that activation of Akt pathway increases CUGBP1 phosphorylation at S-28 in DM1 myoblasts and skeletal muscle, we tested if the increase of CUGBP1 expression observed in our DM cohort is related to an increase of CUGBP1 phosphorylation at S-28. Phosphorylation at S-28 controls nucleus-cytoplasm distribution of CUGBP1, thus it appears that an increase of the expression of CUGBP1-p-S28 isoform may affects CUGBP1 homeostasis since CUGBP1 regulates splicing in the nucleus and stability and translation of mRNA in the cytoplasm, Among all the DM muscles analysed, an increase of CUGBP1-p-S28 was observed only in DM1-E2 which also showed the higher level of CUGBP1 expression. In all other groups, CUGBP1-p-S28 levels are similar to those observed in controls (Fig. 3C). We additionally analysed DM muscle samples through 2D-GE in order to evaluate the CUGBP1 phosphorylation pattern and thus investigate in human biopsies the reported striking protein shift toward a more acidic position previously described in cell system. We analysed CUGBP1 phosphorylation pattern in 12 DM muscle samples confirming an overexpression of CUGBP1 protein only in DM1-E2 respect to control and DM2 biopsies (Fig 3D). In particular, CUGBP1 showed a typical 3 spots pattern in the majority of the sample tested, whereas an additional more acid spot appears only in DM1-E2 sample (Fig. 3D). The appearance of this left spot confirms an increase of the expression of CUGBP1-p form in DM1-E2 patients. Moreover, this additional spot suggests the presence of a more phosphorylated CUGBP1 isoform in DM1-E2, however increased abundance of this isoform should be justified by the overexpression of CUGBP1 in DM1-E2. In order to understand whether CUGBP1 phosphorylation pattern is also altered in DM2 muscles we have compared similar signals of 2D patterns among controls, DM1-E2 and DM2-PROMM to evaluate abundance of different phosphorylation isoforms. As shown in Figure 3E, the CUGBP1 phosphorylation pattern does not show significant alteration in protein spot relative abundance and it does not highlight a hyperphosphorylation of the protein suggesting a similar phosphorylation pattern among the DM phenotype investigated.

CUGBP1 transcript level was higher in both DM1 and DM2 compared to controls however differences were not statistically significant (Fig 3F).

ZNF9/CNBP expression is reduced in DM2 muscle biopsies

We have analysed the expression of ZNF9/CNBP at protein and mRNA levels to verify if there is a relationship between their expression and the DM2 clinical severity. ZNF9/CNBP protein levels in examined DM2 muscles are significantly reduced compared with DM1 and control samples whereas the protein level is similar among DM2 subphenotypes (Fig. 4 A-C). Also *ZNF9/CNBP* mRNA expression appears to be lower in DM2 muscle biopsies than in control biopsies ($p<0,05$; data not shown) and, in agreement with data on protein expression, mRNA levels are similar in the DM2 subtypes considered (Fig. 4D).

IR, CLCN1, SERCA1, MBNL1 and CAPZB alternative splicing alterations are related to clinical severity of DM subphenotypes

In this work we have analysed splicing isoforms of *IR*, *CLCN1*, *SERCA1*, *MBNL1*and *CAPBZ* genes in muscle biopsy from DM patients to understand if a relationship may exist between the degree of splicing alteration and the phenotype severity. Exons inclusion for all these genes is developmentally regulated and

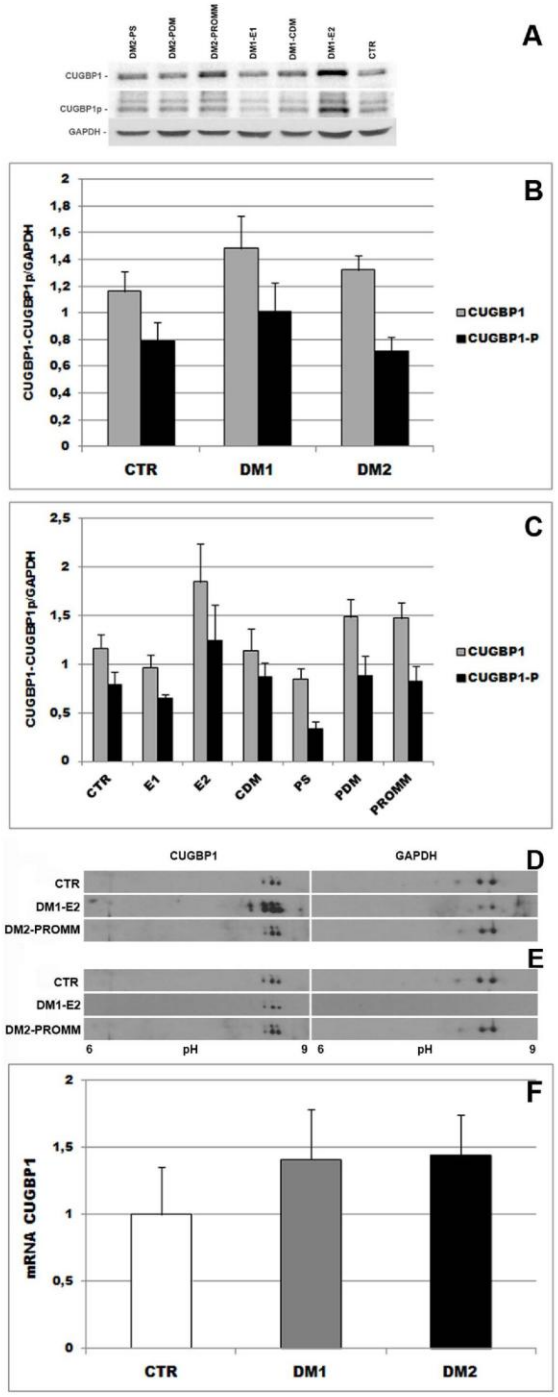

Figure 3. CUGBP1 expression in biceps brachii muscle samples from healthy, DM1 and DM2 patients. A-E. Analysis of CUGBP1 protein expression by western blot. **A**. Representative western blot analysis of CUGBP1 and CUGBP1-p-S28 protein expression in healthy, DM1 and DM2 patients. Density of the bands has been normalized with GAPDH expression used as internal control. **B.** Histograms represents mean values of CUGBP1 protein expression analysed by densitometry in DM1 (n = 18) and DM2 (n = 20) patients compared to controls (n = 8). Bars represent standard error of the mean (SEM). An increase of CUGBP1 and CUGBP1-p-S28 expression is more evident in DM1 muscles. **C.** CUGBP1 expression has been also evaluated in DM1 and DM2 phenotypic subgroups (DM1-E1 (n = 5), DM1-E2 (n = 10), DM1-CDM (n = 3), DM2-PS (n = 5), DM2-PDM (n = 5) and DM2-PROMM (n = 10)) compared to controls (CTR; n = 8). Bars represent SEM. Among all DM muscles analysed, the increase of CUGBP1 and CUGBP1-p-S28 levels is more evident in DM1-E2. **D.** Representative CUGBP1 protein expression pattern of 50 μg of biceps brachii muscle samples from healthy, DM1-E2 and DM2-PROMM patients determined by western blot analysis after 2D-GE separation (left panel). In order to evaluate protein load, GAPDH has been also detected in the 2D map (right panel). **E.** Since CUGBP1 is overexpressed in DM1-E2 (see Figure 3D), different exposition times (2 hours for DM2 and controls *vs* 5 minutes for DM1) have been compared to obtain similar western blot signals with the intent to compare the CUGBP1 phosphorylation patterns (protein distribution among the different CUGBP1 phosphorylated isoforms) in DM1, DM2 and control samples. As illustrated in the left panel, CUGBP1 phosphorylation pattern is not altered in DM1 and DM2 muscles as compared to controls. **F.** CUGBP1 mRNA expression in biceps brachii muscle samples from DM1 (n = 11), DM2 (n = 14) and controls (CTR, n = 4) patients. Bars represent standard deviation.
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dependent on MBNL1 (*SERCA1* and *MBNL1* genes) [53,54], CUGBP1 (*CAPZB* gene) [53] or both MBNL1/CUGBP1 proteins (*CLCN1* and *IR* genes) [55–57]. We have identified similar defects in *IR*, *CLCN1*, *SERCA1*, *MBNL1* and *CAPZB* splicing in DM1 and DM2 where the frequency of abnormal isoforms are significantly increased as compared to controls (Fig. 5A-C). The mean percentage of IR-A isoform (*IR–A/IR–A+IR–B* ratio) in DM1 and in DM2 was 64% and 65% respectively, whereas in controls was 27%. This could explain the insulin resistance in both forms of the disorder. The expression pattern of the *CLCN1* gene has been analyzed across exon 7a, which is abnormally included in DM muscles. In DM1 and DM2 in fact we found 30% and 36% rate of exon 7a inclusion compared to 7% in the control group. *SERCA1* gene is developmentally regulated and SERCA1b isoform, not including exon 22, is characteristic of dystrophic muscle and myotubes [46]. Accordingly to this observation, our DM1 and DM2 samples showed higher level of SERCA1b isoforms than the controls (median percentage of 36% and 22% *vs*. 4% in controls). Similarly, RT-PCR analysis of *MBNL1* splicing pattern across exon 7 region indicated that the ratio of MBNL1 exon 7 inclusion on total *MBNL1* (*MBNLex7/MBNLex7+MBNL1*Δ7) is 52% in DM1, 59% DM2 and 33% in control samples. On the basis of the observed CUGBP1 increased protein levels in DM1 muscle, we also analyzed the expression of the *CAPZB* gene, which encodes for the F actin capping protein beta subunit. *CAPZB* splicing is dependent only on CUGBP1 and is misregulated in DM1 patients [48,54]. RT-PCR analysis showed that the ratio of fetal *CAPZB* exon 8-excluding isoform on total *CAPZB* transcripts (*CAPZBΔ8/CAPZBEx8+CAPZBΔ8*) is 48% in DM1, 37% DM2 and 18% in control groups. Interestingly, the DM1-E2 was the category with the highest levels of *CAPZB* Ex8-exclusion transcripts (59%). When considering single phenotypes, DM1-E1 muscles show a lower frequency of abnormal isoforms than those observed in DM1-CDM and DM1-E2 (Fig. 5D). Also in DM2 group, the degree of expression of pathological isoforms in the paucisymptomatic phenotype appears to be lower than those observed in DM2-PDM and DM2–PROMM (Fig. 5E).

It has been shown that the increase of CUGBP1 contributes to the *IR* and *CLCN1* splicing alteration and is the only factor regulating exon inclusion of the *CAPZB* gene [53,58]. We have found a significant correlation between CUGBP1 protein expression in DM muscles and the frequency of *IR*, *CLCN1* and *CAPZB*

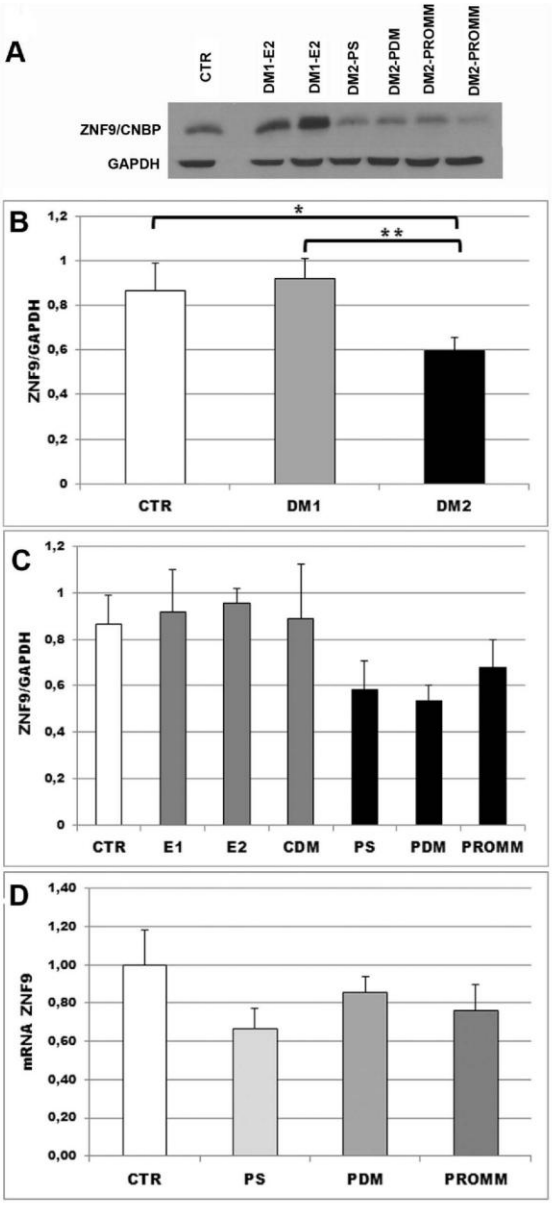

Figure 4. ZNF9/CNBP expression in biceps brachii muscle samples from healthy, DM1 and DM2 patients. A-C. ZNF9/CNBP protein expression determined by western blot analysis. **A.** Representative western blot analysis of ZNF9/CNBP protein expression in healthy, DM1 and DM2 patients. Density of the bands has been normalized with GAPDH expression used as internal control. **B.** Histograms represents mean values of ZNF9/CNBP protein expression analysed by densitometry in DM1 (n = 11) and DM2 (n = 14) patients compared to controls (n = 8). ZNF9/CNBP levels are significantly lower in DM2 muscles as compared to DM1 and control muscles. *$p<0.05$; **$p<0.01$. **C.** ZNF9/CNBP expression has been also evaluated in DM1 and DM2 phenotypic subgroups (DM1-E1 (n = 3), DM1-E2 (n = 5), DM1-CDM (n = 3), DM2-PS (n = 4), DM2-PDM (n = 5) and DM2-PROMM (n = 5)) compared to controls (CTR; n = 8). The expressions levels of the protein are similar in the three DM2 subphenotypes considered. **D.** ZNF9/CNBP mRNA expression in biceps brachii muscle samples from DM2 patients (DM2-PS (n = 4), DM2-PDM (n = 5) and DM2-PROMM (n = 5)) and controls (CTR, n = 3). mRNA levels are similar in DM2 subgroups. Bars represent SEM.
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pathological isoforms. A significant correlation is also evident in DM1 cohort but not in DM2 cohort. The increase of CUGBP1 expression significantly correlate with the splicing alterations observed for *SERCA1* and *MBNL1* although CUGBP1 does not seem to be directly involved in splicing misregulation of these genes.

No correlation has been found between the age of patients and the degree of splicing deregulation of the genes examined both in DM1 and DM2 except for *IR* gene in DM2 cohort ($p<0,01$; data not shown).

Since an elevated concentration of intracellular Ca^{2+} has been suggested to be a possible cause of muscle degeneration [59], we have analysed if there is a correlation between observed histopathological alterations in DM muscles and the expression of pathological isoform of *SERCA1* which is one of the main regulators of intracellular Ca^{2+} homeostasis in skeletal muscle cells. We have found a significant correlation between *SERCA1* splicing alteration and the atrophy factor in DM1 but not in DM2 muscle. A significant correlation between *SERCA1* splicing alteration and hypertrophy factor has been found in DM2.

CLCN1 mRNA expression levels are similar in DM2 subgroups

CLCN1 mRNA expression levels in DM2 muscle biopsies is reduced as compared to control biopsies ($p<0,01$, data not shown) and the mRNA levels appear to be significantly lower in DM2-PDM than in controls (Fig. 6). Statistical analysis does not reveal differences in *CLCN1* mRNA expression levels between DM2 phenotypic subgroups considered in this work (Fig. 6). This feature is confirmed also by splicing analysis with densitometry software where DM2-PS, DM2-PDM and DM2-PROMM present about the same level of pathological isoforms.

Discussion

Myotonic dystrophies are autosomal dominant diseases which share many phenotypic features, however these two disorders also present a number of very dissimilar features making them clearly separate diseases. It is important to underline that DM1 and DM2 phenotypes present a wide clinical spectrum that includes different clinical subphenotypes indicating that molecular pathomechanism of DM is more complex than that actually suggested. The results of our study carried out on skeletal muscle from different DM1 and DM2 subphenotypes seem indicate that the splicing and muscle pathological alterations observed are related to the clinical phenotype. Muscle histopathology of most of DM1 and DM2 patients examined in this work showed the characteristic myopathic features of these diseases. Moreover, in DM2 muscles the mainly affected fiber type is type 2 fibers as previously reported by other authors [29,60,61]. However this is true for DM1 and DM2 patient groups showing the more severe multisystemic phenotypes but not for the groups of paucisymptomatic patients, i.e. DM1-E1 and DM2-PS, who present none or minimal muscle histopathological alterations. Moreover as expected, alteration of alternative splicing of *IR*, *CLCN1*, *MBNL1*, *SERCA1* and *CAPZB* genes is evident in both DM1 and DM2 muscle biopsies despite the clinical phenotype. However it appears that DM1-E1 and DM2-PS patients, who show the less severe clinical and muscle histopathological phenotype, also present a milder spliceopathy profile than those observed in the other DM patients analyzed. It

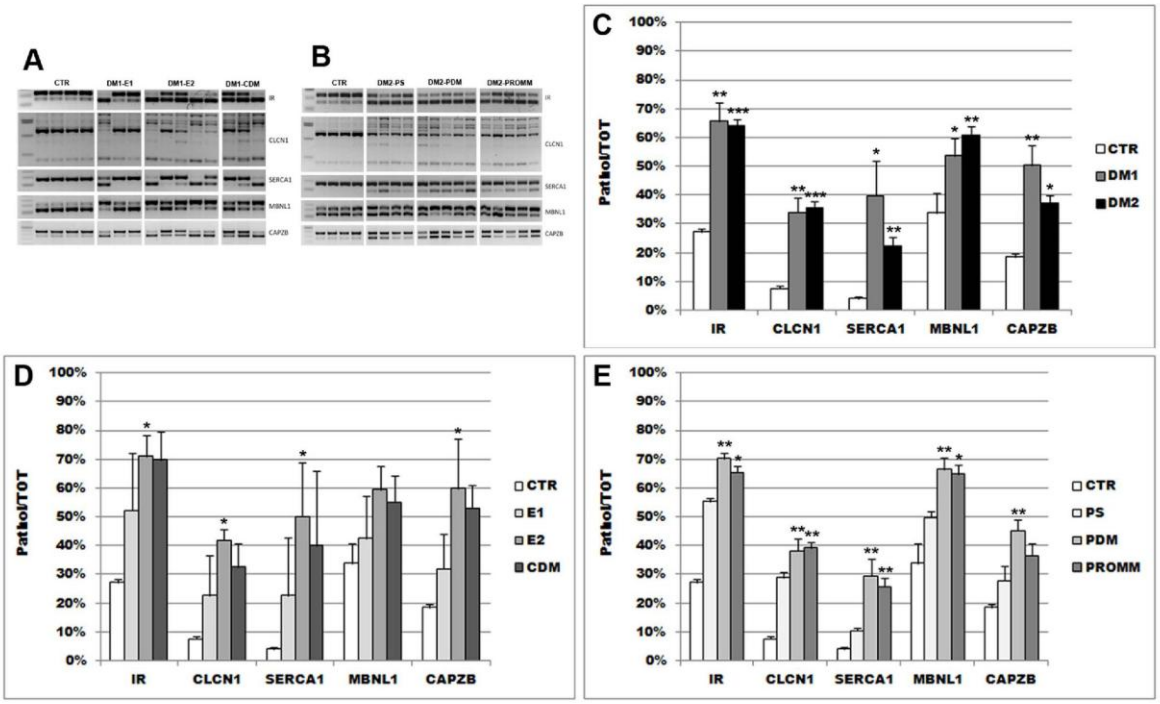

Figure 5. Analysis of alternative splicing of the *IR, CLCN1, SERCA1, MBNL1* and *CAPZB* genes. A, B. Splicing products obtained by RT-PCR amplification of RNA isolated from biceps brachii muscle samples from DM1 (A), DM2 (B) and control patients. **C-E**. Densitometric analysis measuring the fraction of aberrant gene isoforms in DM muscles (**C**), and in DM1 (**D**; DM1-E1 (n = 3), DM1-E2 (n = 5), DM1-CDM (n = 3)) and DM2 (E; DM2-PS (n = 4), DM2-PDM (n = 5), DM2-PROMM (n = 5)) phenotypic subgroups compared to controls (CTR). Bars represent SEM; *p<0.05; **p<0.01; ***p<0.001. Alternative splicing of all five genes analysed appear to be altered in DM as compared to non-DM muscles.
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should be noted that the degrees of *CLCN1* splicing misregulation in DM2-PDM is similar to that observed in DM2-PROMM despite myotonia in DM2-PDM is not evident at clinical level. Also, the *CLCN1* mRNA expression levels appear to be similar in DM2-PDM and DM2-PROMM. However it is possible that symptomatic myotonia is not detectable in DM2-PDM patients due to the high degree of atrophy factor observed in skeletal muscle. It is well known that in DM the clinical myotonia is not present at elevated degree of dystrophy or atrophy. Since an elevated concentration of intracellular Ca^{2+} has been suggested to be a possible cause of muscle degeneration [59], we have analysed if there is a correlation between histopathological alterations observed in DM muscles and the expression of pathological isoform of *SERCA1* which is one of the main regulators of intracellular Ca^{2+} homeostasis in skeletal muscle cells. Both in DM1 and DM2 we have found a positive correlation between the muscle alterations and the *SERCA1* splicing alteration thus strengthen the hypothesis that aberrant splicing of this transcript might contribute to severe histopathological alterations in DM patients.

To better define the molecular pathways which may be involved in disease-specific manifestations, we have analysed the role of CUGBP1 particularly intriguing in DM2 since contradictory results have been reported [34–36]. While it is clear that MBNL1 is depleted from nucleoplasm through recruitment into ribonuclear inclusions both in DM1 and DM2 even when clinical symptoms and muscle alterations are very mild [35,62–65], CUGBP1 overexpression has been clearly demonstrated in DM1 but not in DM2 muscle biopsies. Our western blotting analysis of CUGBP1 protein expression confirms that CUGBP1 is overexpressed in DM1 muscle biopsies however the increase is evident only in DM1-E2 while CUGBP1 protein levels in DM-E1 and

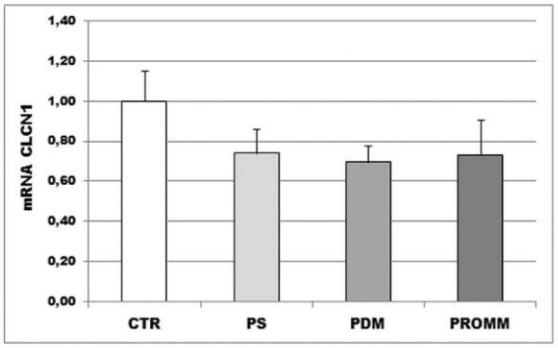

Figure 6. Results of QRT-PCR experiments to quantify the expression level of the *CLCN1* mRNA in biceps brachii muscle samples from DM2 patients (DM2-PS (n = 4), DM2-PDM (n = 5) and DM2-PROMM (n = 5)) and controls. Each experiment has been performed in triplicate and the relative amount of the *CLCN1* transcripts has been determined using the β_2-microglobulin as endogenous control gene. Bars represent standard deviation.
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DM1–CDM appear to be similar to those observed in healthy controls. Moreover CUGBP1 overexpression in DM1-E2 biopsies is accompanied by a parallel increase of the amount of phosphorylated isoform. These data correlate with the splicing analysis of the *CAPZB* gene which is regulated specifically by the CUGBP1 protein.

Except for E1-CDM, our data are in line with those reported by Timchenko et al. [66] on DM1 muscle biopsies. In this work, CUGBP1 appear to be overexpressed in CDM and E2 patients but not in E1 patients. However, only one CDM was examined and no mention was made about the age of the patient.

It has been suggested that in DM1 CUGBP1 may be responsible for muscle wasting since the transgenic mice with skeletal muscle-specific expression of CUGBP1 reproduces the dystrophic muscle histology characteristic of DM1 [67] while MBNL1 knockout mice do not exhibit severe muscle wasting suggesting that MBNL1 depletion alone is not able to reproduce this disease feature [68]. In our work we have found a clear correlation between CUGBP1 expression and the atrophy factors found in DM1 muscles. However when considering the different DM1 clinical phenotypes, DM1-E2 and DM1-CDM show the higher values of atrophy factor and the most severe muscle histopathological alterations nevertheless CUGBP1 is overexpressed only in DM1-E2 muscles. It should be noted that the extreme muscle weakness observed in the congenital form of DM1 is not caused by degenerative changes but by developmental defects. Analysis of muscles from CDM patients has shown that muscle fibers are immature in foetuses and that the skeletal muscle maturation is impaired in children [69,70]. However, the analysis of two successive muscle biopsies of CDM patients showed that in time the muscle is able to gain a certain degree of maturity but never becomes normal since it retains discrepancies in fiber size and the degenerative muscle process begins starting from the second decade when the morphological alterations become identical to those described in late onset myotonic dystrophy [71]. Since we have analysed adult-young CDM patients where muscle histopathological alterations might be due more to the developmental defects than to the degenerative process, it is possible that CUGBP1 expression in our DM1-CDM muscles appears to be similar to DM1-E1 more than to DM1-E2.

Contrary to DM1, in DM2 muscle biopsies examined in this work a slight increase of the CUGBP1 protein levels is observed in DM2-PDM and DM2-PROMM but not in DM2-PS However this increase is not related to an increase of protein phosphorylation. In addition our data on DM2 muscle seem suggest that perturbation of CUGBP1 amount are not required to produce histopathological or splicing regulation defects in DM2. We have observed that the greater expansion in DM2 leads to ribonuclear foci greater than in DM1 which can sequester larger amount of MBNL1. Therefore, the depletion of MBNL1 from nucleoplasm appears to be more extensive in DM2 than in DM1 despite DM1 shows a greater severity of the muscle degeneration (unpublished data). Thus, since sequestration of MBNL1 evidently has a central role in splicing misregulation in both types of DM, it appears likely that in DM1 CUGBP1 overexpression might be an additional pathogenic mechanism not shared by DM2.

It is relevant to highlight that we do not find differences in phosphorylation pattern between the DM phenotypes suggesting that CUGBP1 does not result hyperphosphorylated in DM compared to control muscles. The discrepancy observed between our data on CUGBP1 expression/phosphorylation in DM muscles and those reported by other Authors may be accounted for the model used: measurements made in cultured cells or in animal models which have been used to induce DM pathomechanism may be different from results obtained in human muscle *in vivo*. Moreover differences may exist between different muscle types used.

It has been suggested that also the reduction of ZNF9/CNBP expression in DM2 patients may explain some of the phenotypic disparities between both types of DM. It has been shown that reduction of ZNF9/CNBP levels is sufficient to produce multiorgan symptoms resembling those of DM as observed in heterozygous $Znf9^{-/-}$ knockout mice [72]. We have determined that ZNF9/CNBP protein and mRNA levels in muscle biopsies of biceps brachii from DM2 patients are significantly reduced compared with non-DM2 individuals, including patients with DM1. Our findings are consistent with recent reports of reduced ZNF9/CNBP expression in DM2 [36,39,40] and these data indicate that ZNF9/CNBP expression might play a role in phenotypic differences between DM1 and DM2. However ZNF9/CNBP protein appears to be equally expressed in the three DM2 phenotypic groups examined in our work, thus ZNF9/CNBP expression levels do not explain the extreme variability of clinical phenotype evident among DM2 patients. Indeed the expression of ZNF9/CNBP protein in DM2-PS is similar to those observed in DM2-PROMM and DM2-PDM despite paucisymptomatic patients show minor muscle histopathological alterations and the frequency of abnormal isoforms of the genes analysed is lower than in symptomatic patients.

This is the first study on a large number of muscle biopsies from DM1 and DM2 patients analysed at histopathological and biomolecular level. Our results indicate that CUGBP1 seems to play a role in classic DM1 more evidently than in DM2 however no definitive conclusions can be drawn due to the high interindividual variability observed in the different parameters analysed in this study. Nevertheless, it appears that the multisystemic disease spectrum and the phenotypic variability of DM pathologies may not be explained only by spliceopathy thus confirming that the molecular pathomechanism of DM is more complex than that actually appreciate.

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Author Contributions

Conceived and designed the experiments: RC EB AB GM. Performed the experiments: RC EB LVR GR GC RV. Analyzed the data: RC EB AB GM. Contributed reagents/materials/analysis tools: RC GM. Wrote the paper: RC EB AB GM. Critical discussion of the data and revision of the manuscript: RC EB LVR GR GC RV GN AB GM.

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Progression of muscle histopathology but not of spliceopathy in myotonic dystrophy type 2

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Abstract

Myotonic dystrophy type 2 (DM2) is an autosomal dominant progressive disease involving skeletal and cardiac muscle and brain. It is caused by a tetranucleotide repeat within the first intron of the *CNBP* gene that leads to an alteration of the alternative splicing of several genes. To understand the molecular mechanisms that play a role in DM2 progression, the evolution of skeletal muscle histopathology and biomolecular findings in successive biopsies have been studied. Biceps brachii biopsies from 5 DM2 patients who underwent two successive biopsies at different years of age have been used. Muscle histopathology has been assessed on sections immunostained with fast or slow myosin. FISH in combination with MBNL1-immunofluorescence has been performed to evaluate ribonuclear inclusion and MBNL1 foci dimensions in myonuclei. Gene and protein expression and alteration of alternative splicing of several genes have been evaluated over time. All DM2 patients examined show a worsening of muscle histopathology and an increase of foci dimensions over time. The progressive worsening of myotonia in DM2 patients may be due to the decrease of *CLCN1* mRNA observed in all patients examined. However, a worsening of alternative splicing alterations has not been evidenced over time. The data obtained in this study confirm that DM2 is a slow progression disease since histological and biomolecular alterations observed in skeletal muscle are minimal even after 10-year interval. The data indicate that muscle morphological alterations evolve more rapidly over time than the molecular changes thus indicating that muscle biopsy is a more sensitive tool than biomolecular markers to assess disease progression at muscle level.

Keywords: Myotonic dystrophy type 2; Ribonuclear inclusions; Disease progression; Muscle histology; Alternative splicing

1. Introduction

Myotonic dystrophy type 2 (DM2) is characterized by autosomal dominant progressive myopathy, myotonia and multiorgan involvement [1–3]. DM2 mutation consists in the expansion of an unstable CCTG tetranucleotide within the first intron of the CCHC-type zinc finger, nucleic acid-binding protein (*CNBP*) gene [4] on chromosome 3q21 and is thus similar to the untranslated (CTG)n repeat in the *DMPK* gene causing DM type 1 (DM1) [5,6]. The prevailing paradigm is that both disorders are toxic RNA diseases since

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CUG/CCUG-containing mutant transcripts aggregate in cell nuclei as ribonuclear inclusions that alter the activity of specific RNA-binding proteins such as MBNL1 and CUGBP1 [7–9]. This results in a "spliceopathy", i.e. alteration of alternative splicing of several downstream effector genes, which is thought to account, at least in part, for multiorgan involvement [10,11]. At skeletal muscle level, DM2 has been defined as "a disease of type 2 fibers" since these myofibers are selectively affected by atrophy and central nucleation [12,13]. The definitive diagnosis of DM2 can be achieved through the detection of the DM2 mutation either at DNA or RNA level with the identification of the expanded RNAs as ribonuclear inclusions by in situ hybridization [14–16]. The progression of the DM2 is slow and prognosis and life expectancy are more favorable than in DM1. Life expectancy may be reduced only in single DM2 cases, mainly if there is severe cardiac involvement with malignant rhythm abnormalities. Thus DM2 is considered a clinically milder disease than DM1. While DM2 disease progression is well documented by clinical reports mainly on cardiac, central nervous system and neuromuscular involvement [17,18], no literature data are available on correlations between muscle involvement progression with biomarkers for disease severity. Therefore in this work we have studied the progression of the muscular involvement in relation to the evolution of skeletal muscle histopathology and biomolecular findings to better prognosticate patients with DM2. Despite the limited number of patients examined, this study may contribute to better understand the molecular mechanisms underlying DM2 pathology.

2. Materials and methods

This study was authorized by the Institutional Ethics Committee (ASL MI2-Melegnano via VIII Giugno, Milan) and was conducted according to the principles expressed in the Declaration of Helsinki, the institutional regulation and Italian laws and guidelines. All blood samples and muscle biopsies were used for this study after receiving written informed consent from the patients.

2.1. Patients

Human muscle biopsies were taken under sterile conditions from 5 DM2 patients who underwent two successive biceps brachii biopsies from the same side at different years of age. Biceps brachii biopsies from two patients with classical DM1 phenotype and from two healthy subjects were also used. The diagnosis of DM was based upon the clinical diagnostic criteria set by the International Consortium for Myotonic Dystrophy [19]. Muscle strength was measured using the modified 5-point MRC scale (Medical Research Council) in the upper and lower limbs for a total of 150 maximum score. Basal EKG and echocardiographic evaluation was performed in all patients.

2.2. Molecular analysis of the CCTG expansion in the CNBP gene

Genomic DNA has been extracted from muscle homogenates with a salting-out procedure [20]. Detection of the CCTG expansion has been obtained with a Long-PCR based method as previously described [16].

2.3. Muscle histopathology

Muscle tissue was fresh-frozen in isopentane cooled in liquid nitrogen. Histopathological analyses were performed on serial sections (8 μm) processed for routine histological or histochemical stainings. A standard myofibrillar ATPase staining protocol was used after preincubation at pH 4.3, 4.6, and 10.4 [21]. The most typical alterations, such as nuclear clump fibers (i.e. aggregates of myonuclei with a thin rim of cytoplasm), nuclear centralization and fiber size variability were evaluated on serial muscle sections.

2.4. Immunohistochemistry

Serial transverse muscle cryostat sections 6 μm thick were cut for immunohistochemical staining (IHC). Sections were air-dried and rehydrated in phosphate buffer pH 7.4 (PBS). Non-specific binding sites were blocked with normal goat serum (NGS; Dako, Denmark) at a dilution 1:20 in PBS containing 2% bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO) for 20 min at room temperature (RT). Mouse monoclonal primary antibodies against two different myosin heavy chain (MHC) isotypes were used at the following dilutions: MHfast, 1:400 in PBS + 2% BSA; MHCslow, 1:400 in PBS + 2% BSA (Sigma–Aldrich, St. Louis, MO). Each antibody was applied for 1 h at RT. After washing in PBS 3 times for 5 min, sections were incubated with goat anti-mouse biotinylated secondary antibody diluted 1:300 in PBS + 2% BSA for 1 h at RT. After PBS washing (3 × 5 min), sections were incubated with Vectastain Elite ABC kit (Vector Laboratories; Burlingame, CA) for 30 min and then exposed to the 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich, St. Louis, MO) chromogen reaction solution for 10 min. Nuclei were counterstained with Mayer's haematoxylin. Quantitative evaluation of fiber diameter was made as described previously by Vihola et al. [12] on images with Image J (Scion Corporation, Frederick, MD) on images taken with a light microscope (160×, original magnification). The size of muscle fibers was assessed by measuring the "smallest fiber diameter". All data were elaborated using Microcal Origin (Microcal Software Inc., Northampton, MA, USA). The metahistograms were normalized to normal mean diameter for men and

women. Atrophy and hypertrophy factors were also calculated [21].

2.5. Fluorescence in situ hybridization (FISH) combined with MBNL1 immunofluorescence

FISH was performed on DM1 and DM2 muscle frozen sections using a $(CAGG)_5$ or $(CAG)_6$-CA probe (IDT, Coralville, IA, USA) as previously reported by Cardani et al. [14] to verify the presence of ribonuclear inclusions. In brief, 6 μm thick transverse cryostatic sections were air dried for 30 min and fixed with 2% paraformaldehyde for 30 min at 4 °C. The sections were then washed in PBS and permeabilized for 5 min in 2% acetone in PBS, pre-chilled at −20 °C. After washing in PBS, sections were incubated in 40% formamide and 2× saline solution citrate (SSC) for 10 min at RT, and hybridized for 2 h at 37 °C, with 1 ng/μL probe $(CAGG)_5$ or $(CAG)_6$-CA Texas red labeled probes in 30% formamide, 2× SSC, 0.02% BSA, 67 ng/μL yeast tRNA, 2 mM vanadyl ribonuclease complex (all these reagents were from Sigma–Aldrich, St. Louis, MO). The sections were washed first in 40% formamide and 2× SSC at 45 °C for 30 min, then in 1× SSC at 45 °C for 15 min and another 1× SSC wash at RT. The FISH-labeled sections were then processed for the immunofluorescence detection of MBNL1, as follows. The sections were pre-incubated for 20 min at RT with 5% NGS in PBS containing 2% BSA and then incubated overnight at 4 °C with a rabbit polyclonal antibody recognizing MBNL1 (kind gift of Prof. C.A. Thornton, University of Rochester, New York, USA); the primary antibody was diluted 1:1000 in PBS containing 2% BSA. The sections were washed in PBS and then incubated for 1 h at RT with a goat anti-rabbit Alexa488-labeled antibody (Molecular Probes, Life Technologies, Milan, Italy), diluted 1:200 in PBS, counterstained for DNA with DAPI (1 μg/ml; Sigma–Aldrich, St. Louis, MO), and mounted with Mowiol (Calbiochem, Milan, Italy). As controls, some slides were processed as described above but omitting the incubation with the primary antibody.

2.6. Morphometric evaluation of the ribonuclear inclusions and MBNL1 foci

RI and MBNL1 foci dimensions have been measured in patients #1, #2, #3 and #5. Double-labeled sections by FISH and MBNL1 were analyzed by confocal microscopy, using a Leica TCS SP2 AOBS system: for fluorescence excitation, an Ar/Vis laser at 488 nm for Alexa488, and He/Ne laser at 543 for Texas red; the laser intensity, pinhole opening, signal amplification and image spacing along the z axis were kept constant, and images were recorded in the 1024 × 1024 pixels format, using a 63× oil immersion objective. For each section, 60 ribonuclear inclusions and the corresponding MBNL1-containing foci were measured. Acquisitions were carried out at the Centro Interdipartimentale di Microscopia Avanzata (CIMA) of the University of Milan, Italy. The size and fluorescence intensity of the ribonuclear inclusions and MBNL1 foci were measured using the Leica Confocal Software, and the mean values and standard deviations were calculated.

2.7. Western blot analysis

Whole cell extracts were obtained from fifteen-twenty consecutive muscle cryostat sections 10 μm thick homogenized in 60 μl of 50 mM TrisHCl with 5% SDS (pH 7.5). After incubating on ice for 15 min, samples were centrifuged at 5700g for 12 min at 4 °C, and supernatant was collected and stored at −80 °C. Pellets were resuspended in 50 mM TrisHCl with 5% SDS (pH 7.5) and stored at −80 °C. Protein concentration in each sample was determined by using BCA Protein Assay (Bio-Rad Laboratories, Milano, Italy). An equal amount of protein was loaded per lane and electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gels, and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Milano, Italy). After blocking non specific sites in TrisHCl buffer pH 7.5 (TBS) containing 5% BSA for 30 min at 42 °C, membranes were incubated overnight at 4 °C with mouse monoclonal anti CUGBP1 (clone 3B; 1:1000; SantaCruz Biotechnology, Heidelberg, Germany) or with rabbit polyclonal anti-ZNF9/CNBP (1:1000) [22]. After several washes in TBS + 0.2% Tween20 or TBS + 0.3% Tween20 respectively, membranes were incubated with horseradish peroxidase-conjugated (Jackson ImmunoResearch Laboratories, Baltimore, PA) goat anti-mouse (1:5000 in TBS + 5% BSA + 0.2% Tween20) or goat anti-rabbit (1:10,000 in TBS + 5% BSA + 0.2% Tween20) secondary antibodies. Membranes were washed and immune complexes were detected using the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ). GAPDH (polyclonal antibody diluted 1:80,000; Sigma–Aldrich, St. Louis, MO) was used as internal control to verify and correct for loading error. Blots have been performed in triplicate.

2.8. Study of alternative splicing

Frozen muscle samples were practiced for the extraction of total RNA using TRIzol reagent (Gibco BRL, Gaithersburg, MD) and 1 μg of RNA was reverse transcribed according to the cDNA protocol of the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Splicing pattern profile of the *IR*, *CLCN1*, *MBNL1* and *SERCA1* genes was carried out as described [23–25]. Total PCR products, obtained within the linear range of amplification, were electrophoresed on 2.5% agarose gel. Quantitative analysis of the amplified products was performed using SybrGreenII-stained gels (Perkin-Elmer Life Science, Massachusetts, USA) scanned on a fluorimager 595 (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band and

the fraction of abnormally (or pathologically) spliced (AS) isoforms (AS-isoforms/total) were quantified by densitometry using ImageQuant software. Control of the RT-PCR reaction was based on the expression level of the glucose phosphate isomerase housekeeping gene (GPI) and all amplifications have been carried out in triplicate using independent cDNA samples.

2.9. QRT-PCR expression analysis of the CLCN1 and CNBP genes

Following RNA extraction and retro-transcription, cDNAs of DM2 samples were also used to quantify the expression level of the *CLCN1* and *CNBP* genes. The total expression of mentioned genes was evaluated using specific TaqMan gene expression assays: *CLCN1* [Hs00163961_m1] and *CNBP* [Hs00231535_m1] (Applied Biosystems, Foster City, CA). The VIC-labeled β_2-microglobulin gene (*B2M*: GenBank accession #NM_004048) was used as housekeeping internal control gene, as described [26]. The simultaneous measurement of genes-FAM/B2M-VIC expression allow to normalize the amount of cDNA added per sample. Each PCR reaction was performed in triplicate using the TaqMan Universal PCR Master Mix and the ABI PRISM 7500 Fast System (Applied Biosystems, Foster City, CA). A comparative threshold cycle (Ct) was used to determine gene expression compared to a calibrator (median value of control subjects). Hence, steady-state mRNA levels were expressed in *n*-fold difference relative to the calibrator. For each sample, genes' Ct value was normalized using the formula $\Delta Ct = Ct\ genes - Ct\ B2M$. To determine the relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct\ sample - \Delta Ct\ calibrator$. The value adopted to plot relative gene expression was calculated using the expression $2^{-\Delta\Delta Ct}$.

2.10. Statistical analysis

The Student's *t*-test or the non parametric Mann–Whitney test has been performed to determine significant differences between the two successive muscle biopsies in each patient.

3. Results

3.1. Patients

Clinical data on DM2 patients used in this study are reported in Table 1. Patient #1 was evaluated at the age of 35. She presented a severe weakness conditioning gait difficulty. Both myotonia and myalgia were reported. At the second evaluation, 10 years later, a further deterioration of muscle strength and a mild decrease of myotonia were evidenced. At the first examination patient #2 (33 yr old) reported stiffness and myalgia since he was 19 years old. Neurological examination showed normal strength and trophism. Myotonia with warm up phenomenon was evidenced. Five years later, when the second biopsy was performed, the clinical phenotype was unchanged and the patient showed a clinical improvement of myotonia and myalgia due to mexiletine treatment. Cardiac parameters were stationary. Patient #3 started to complain of proximal leg weakness at the age of 36 and he was evaluated for the first time at the age of 54. He presented widespread myalgia, mild hand percussion myotonia and proximal weakness both to the upper and lower limbs. During the second evaluation, 4 years later, he reported an improvement of myotonia and myalgia due to mexiletine and a worsening of proximal weakness. Cardiac ECG showed the appearance of a BAV I grade block (PR > 200 ms). Patient #4 was visited twice, first at 58 and then at 60 years of age. The clinical findings were comparable and were characterized by proximal weakness, moderate hand myotonia and myalgia. Finally, patient #5 complained muscle pain since he was 30 years old. The first biopsy was performed at the age of 51. At that time he presented grip myotonia, myalgia and proximal weakness. He was admitted to our department for a second biopsy 2 years later with an unaltered clinical situation.

Table 1
Clinical data on DM2 patients used in this study.

Pt	#1	#2	#3	#4	#5
Time between evaluation	10	5	4	2	2
Age baseline	35	33	54	58	51
Age follow-up	45	38	58	60	53
MRC[a] muscle biopsied Baseline	3	5	4.3	5	4
MRC muscle biopsied follow-up	2	5	4	5	4
MRC total baseline	93	150	139.3	145	143
MRC total Follow-up	88	150	136	145	143
PR baseline	140	161	118	152	172
PR follow-up	144	156	210	172	168
QRS baseline	85	104	86	104	116
QRS follow-up	92	100	106	106	116
FE% baseline	63.5	76.5	62.2	n.d.	65.8
FE% follow-up	63	70	66.7	56.4	61.4

[a] Medical Research Council, scale for muscle strength; scale (0–5 grade) on 15 muscles at both side in the upper and lower limbs for a total of 150 maximum score.

3.2. Muscle histopathology

All DM2 patients examined show a worsening of muscle histopathology over time (Figs. 1 and 2). Central nucleation is always present involving prevalently type 2 fibers (Figs. 1A–C) and the percentage of type 2 fibers presenting central nucleation increases in the second biopsy compared to the first one (Fig. 1C). The majority of nuclear clumps fibers present in the muscle sections examined, express only the MHC fast myosin, while nuclear clumps fibers expressing only MHC slow myosin are rarely or no present (Fig. 1A and B). The number of MHCfast positive nuclear clumps appears to be higher in the second biopsy than in the first one in all patients (Fig. 1D). The metahistograms based on the analysis of fiber diameters on immunostained muscle sections and the evaluation of atrophy (AF) and hypertrophy (HF) factors are reported in Fig. 2. Analysis of MHC fast and slow myosin immunostained muscle sections allows us to detect and measure fibers smaller than 5 μm, including all nuclear clump fibers which are recognizable by the presence of a thin rim of immunoreaction around the nuclei. An increase of both type 1 and type 2 fiber AF is evident between the first and the second biopsy in all patients examined. However, as expected in DM2 muscle, atrophy is more marked in type 2 than type 1 fibers, except for one patient (Pt #2) presenting a very mild phenotype, where type 1 and type 2 fiber atrophy appears to be similar in both biopsies (Fig. 2).

3.3. Ribonuclear inclusions (RI) and MBNL1 foci dimensions

RI and MBNL1 foci dimensions have been measured on images obtained at confocal microscopy of the two successive DM2 muscle biopsies to verify if an increase of foci dimensions is evident over time. As expected, a clear co-localization of ribonuclear inclusions and MBNL1 foci is evident in all the muscle biopsies examined. In all DM2 patients considered, areas of RI or MBNL1 foci (Fig. 3) and the corresponding fluorescence intensities (data not shown) show a significant increase of both foci type dimensions in the second biopsy compared to those observed in the first one. However the fold increase of foci dimensions does not appear to correlate with the time elapsed between the two successive biopsies. Interestingly, the RI and MBNL1 foci in DM2 muscle appear to be significantly larger than those observed in DM1 muscle nuclei (Fig. 3E–I).

3.4. Western blotting analysis

The protein levels of CUGBP1 and CNBP have been evaluated in the two successive muscle biopsies from DM2 patients by western blotting analysis. CUGBP1 protein level is higher in the second biopsies in all the patients examined (Fig. 4A and B). The most evident increase of CUGBP1 expression between the first and the second biopsy (4.7-fold increase) is observable in patient

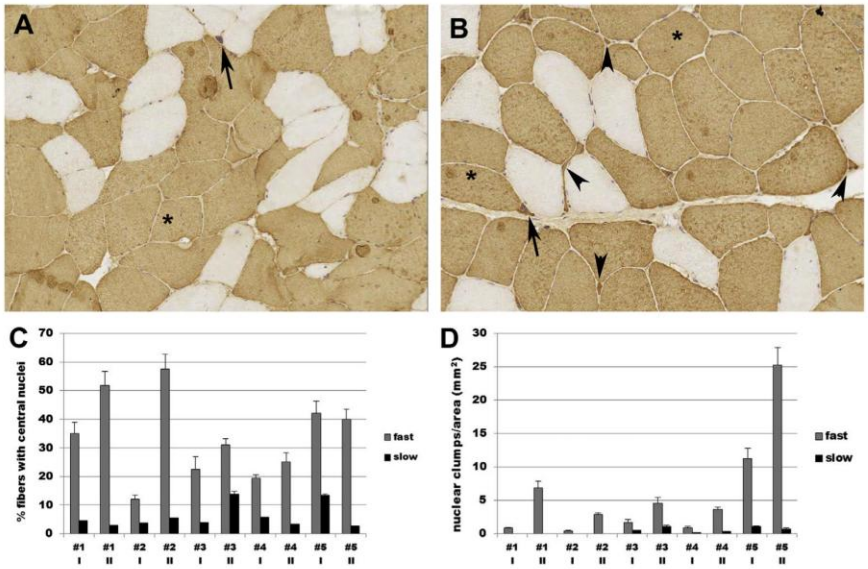

Fig. 1. Fast myosin immunostaining of skeletal muscle transverse sections obtained from the first biopsy (A) and from the second biopsy (B) of patient #4. Type 2 fibers (fast positive fibers) are stained in brown. Note the increase of the number of very atrophic fibers fast positive (arrowheads) and of type 2 fiber with central nuclei (asterisks). Arrows indicate type 2 nuclear clumps. Original magnification 400×. The percentage of fibers with central nuclei (C) and the number of nuclear clumps per area (D). Histograms represent the mean ± SD of quantifications made on three different sections for each biopsy.

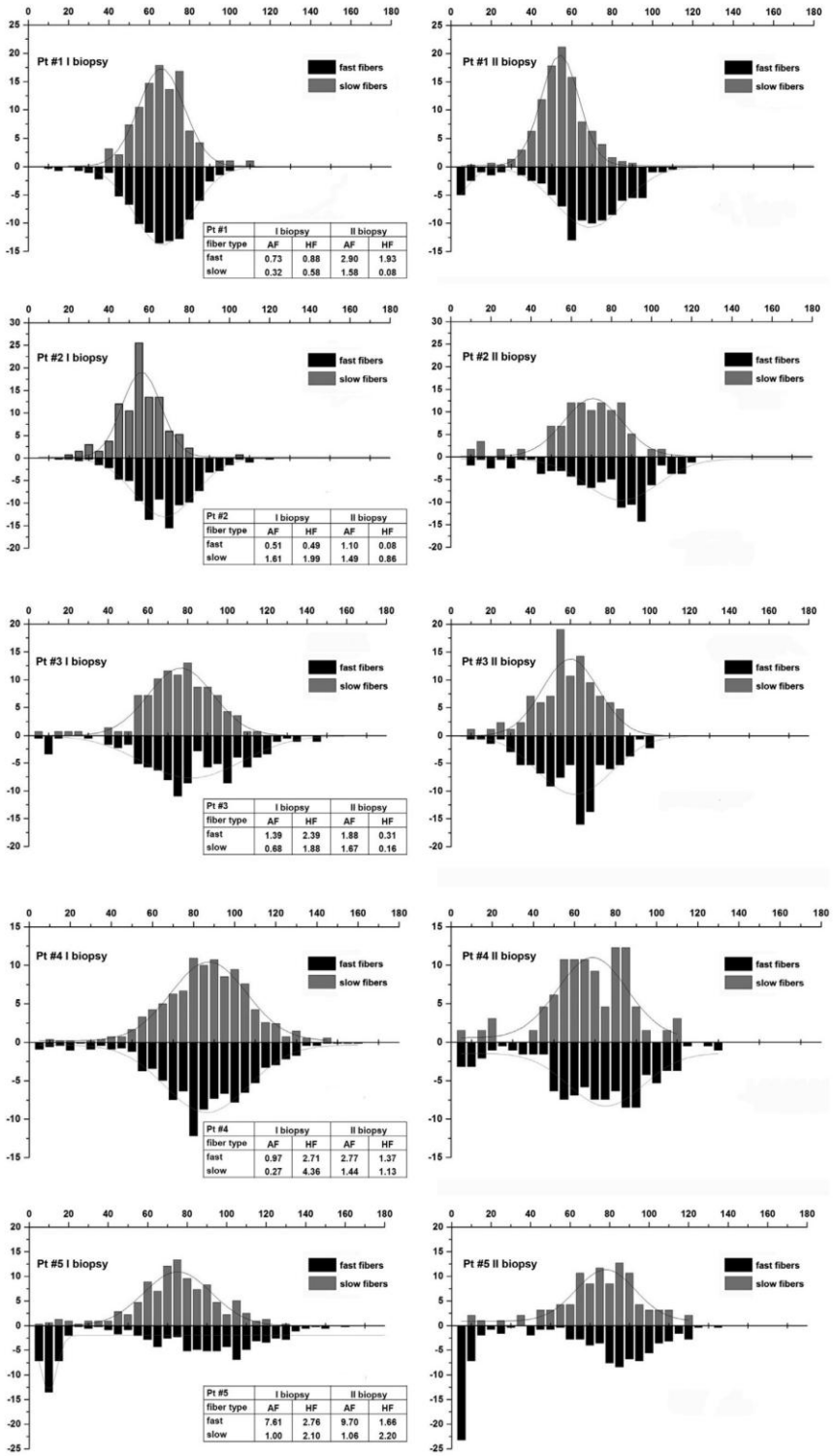

Fig. 2. Metahistograms obtained from the analysis of muscle fiber diameters in the first (left) and second (right) muscle biopsy from 5 DM2 patients. The results are based on sections immunostained for MHC fast or slow myosin. Tables show the relative atrophy or hypertrophy factors in both biopsies of each patient.

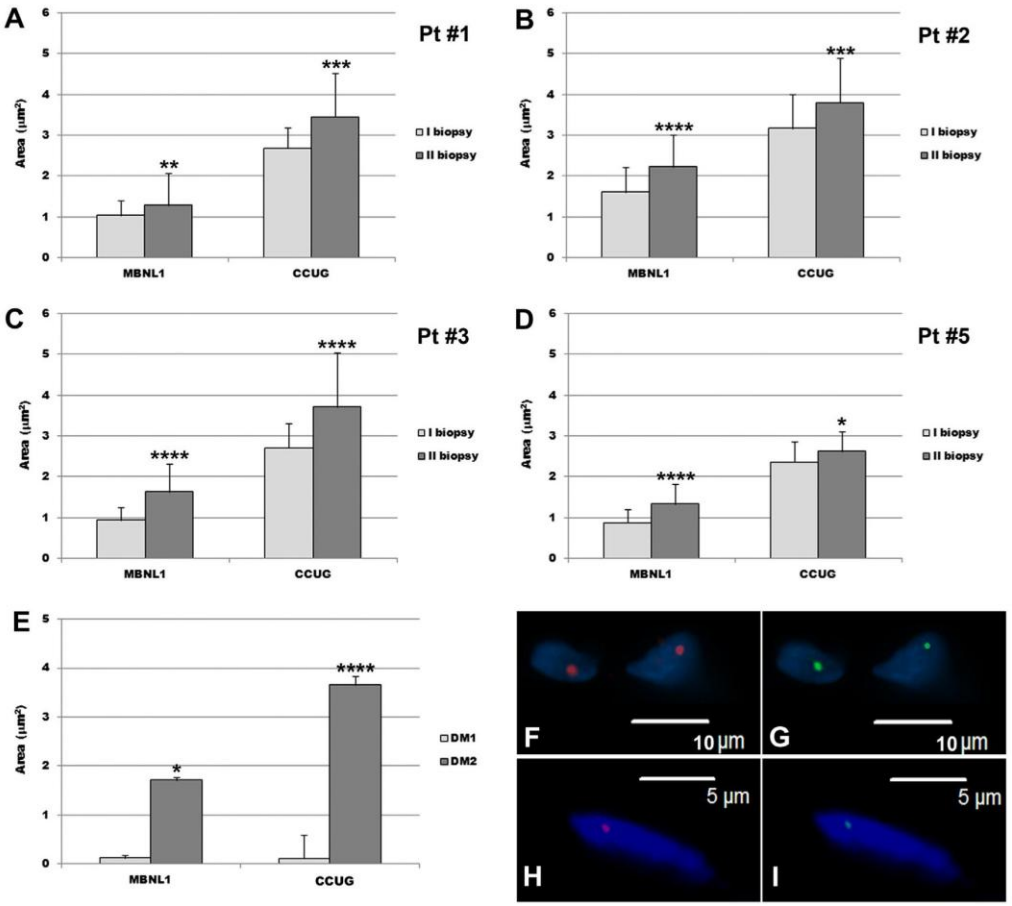

Fig. 3. Ribonuclear inclusions and MBNL1 foci dimensions in patients #1 (A), #2 (B), #3 (C) and #5 (D). Histograms indicate the mean ± SD of area of ribonuclear inclusions and MBNL1 nuclear foci obtained at confocal laser scanning microscopy in the first and second biopsy. Comparison between foci dimensions in 2 DM1 patients and those observed in 2 DM2 patient (#1 and #3, second biopsy) (E). FISH in combination with MBNL1 immunofluorescence performed on DM2 muscle (F and G) and on DM1 muscle (H and I) shows that ribonuclear inclusions (F and H; red spots) and MBNL1 foci (G and I; green spots) colocalize in nuclei (blue, DAPI) and appear to be larger in DM2 than in DM1. Mann–Whitney test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

#1 whose second biopsy was performed after 10 years from the first one (Fig. 4B). CUGBP1 expression in DM2 muscle biopsies appears to be similar or lower than those observed in control biopsies (Fig. 4A).

CNBP protein levels in DM2 muscles examined are similar in the two successive biopsies and appear to be reduced compared with control samples (Fig. 4C and D).

3.5. Alternative splicing

In this work we have analyzed splicing isoforms of *IR*, *CLCN1*, *SERCA1* and *MBNL1* genes in two successive muscle biopsies from DM2 patients to understand if a worsening of clinical phenotype is associated to a worsening of alternative splicing alteration of these genes in DM2 skeletal muscle. As expected, a statistically significant alteration of alternative splicing of the genes examined in this work is present in all the DM2 muscle biopsies considered as compared to controls (Fig. 5). However, no difference or a slight increase in the expression of *IR* and *MBNL1* pathological isoforms is observable between the first and the second biopsy of all the patients examined (Fig. 5). *SERCA1* and *CLCN1* genes splicing pattern is however more variable and appears worsened in patients #3, #4 and #5 with no obvious correlation with the muscle phenotypes (Fig. 5). No correlation is evident between the variation of spliceopathy and the time elapsed between the two successive biopsies.

3.6. CLCN1 and CNBP mRNA expression level

CLCN1 mRNA expression level shows a decrease in the second biopsy of all the patients examined. However this reduction appears to be statistically significant only for patient #1 whose second biopsy was performed 10 years

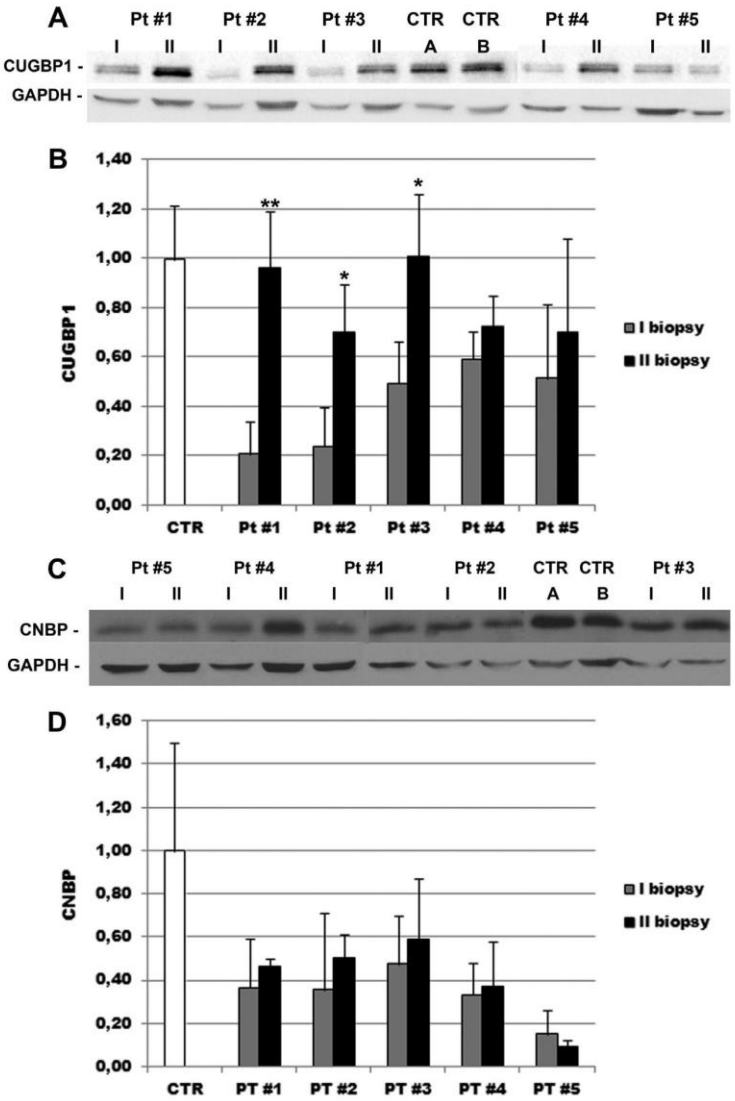

Fig. 4. Western blot analysis of CUGBP1 and CNBP protein expression in muscle samples from 2 healthy subjects and from two successive biopsies of 5 DM2 patients. Density of the bands has been normalized with GAPDH expression used as internal control (A and C). Histograms represent mean values ± SD of CUGBP1 and CNBP protein expression analyzed by densitometry (B and D). Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$.

after the first one and for patient #5 who presents the most severe muscle histopathology (Fig. 6A). *CNBP* mRNA expression appears to be similar in the two successive biopsies in all patients examined except for patient #1 who presents a significant decrease of mRNA level in the second biopsy performed 10 years after the first one (Fig. 6B).

4. Discussion

The expansion of the CCTG repeat in the first intron of *CNBP* gene results in a spliceopathy of downstream effector genes which is thought to account, at least in part, for multiorgan involvement [7,8]. At clinical level it has been observed that in DM2 as well as in DM1 patients, symptoms such as muscle weakness and myotonia undergo progressive worsening with increasing age [27]. Moreover, alternative splicing changes in skeletal muscle has been correlated with muscle weakness to some extent both in DM1 and DM2 patients [28]. We therefore studied the progression of DM2 through the analysis of histological and biomolecular disease markers in muscle biopsy. Our analysis of two successive biopsies of the same patient indicates a clear worsening of muscle histopathology over time and this worsening mostly occurs in type 2 fibers. Muscle degeneration appears to be more pronounced after a 10-year interval between the two successive biopsies; however it is already evident

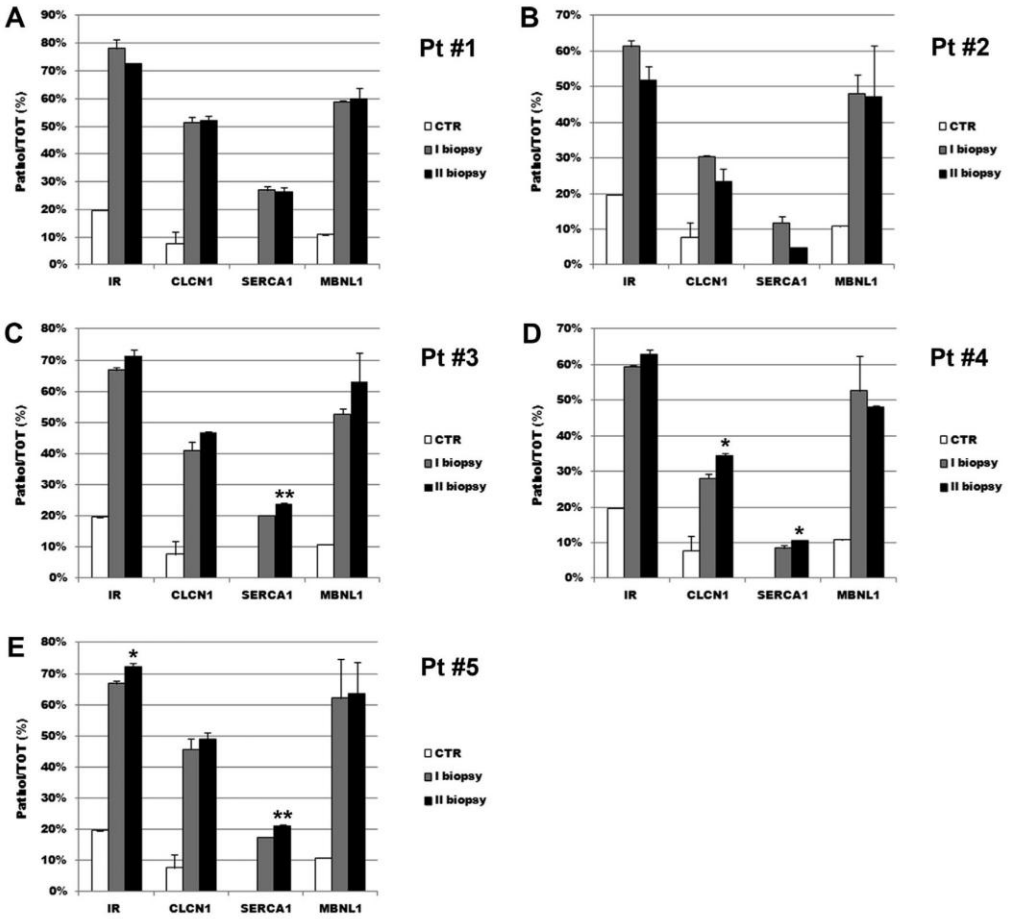

Fig. 5. Splicing analysis of the *IR*, *CLCN1*, *SERCA1* and *MBNL1* genes in muscle samples from 2 control subjects and from two successive biopsies of 5 DM2 patients (A–E). Histograms represent mean ± SD. Student *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$.

after 2 years. This degenerative process may explain the worsening of muscle symptoms like weakness and wasting and suggests that muscle biopsy represents a good tool to follow the progression of the disease. It has been reported that the expansion of DNA repeats increased by approximately 2 kb in the leukocytes during the 3-year interval between two successive blood donations [4,29,30]. In this study, because of the low amount of genomic DNA extracted from muscle tissue, we detected the DM2 mutation by a qualitative long-PCR assay, which does not allow the estimation of the expansion size. However, our evaluation of dimensions of ribonuclear inclusions and MBNL1 foci demonstrates that both foci types increase in size over time in DM2 myonuclei indicating that CCUG repeats expand in skeletal muscle as well as in blood with increasing age and that a longer CCUG repeat is associated with sequestration of a larger amount of MBNL1, as previously reported for CUG repeat [31]. Thus the worsening of muscle symptoms with aging in DM2 patients may be caused, at the cellular level, by the progressive enlargement of repeats and by the consequent increase of the sequestration of factors involved in RNA processing and export to the cytoplasm. However, DM2 ribonuclear inclusions appear to be larger than DM1 and sequester a larger amount of MBNL1. Therefore, the depletion of MBNL1 from nucleoplasm seems to be more extensive in DM2 than in DM1 despite DM1 is considered a more severe disease. It appears likely that in DM1an additional pathogenic mechanism not shared by DM2 may exist, since sequestration of MBNL1 evidently has a central role in splicing misregulation in both types of DM. It has been reported that in DM1 a combined effect of decreased MBNL1 and increased CUGBP1 activity leads to misregulated alternative splicing and other changes of the muscle transcriptome [25,32], however evidence that CUGBP1 upregulation also occurs in DM2 is conflicting [31–33]. Our data indicate that CUGBP1 protein is not overexpressed in DM2 muscle biopsies as compared to controls as previously reported

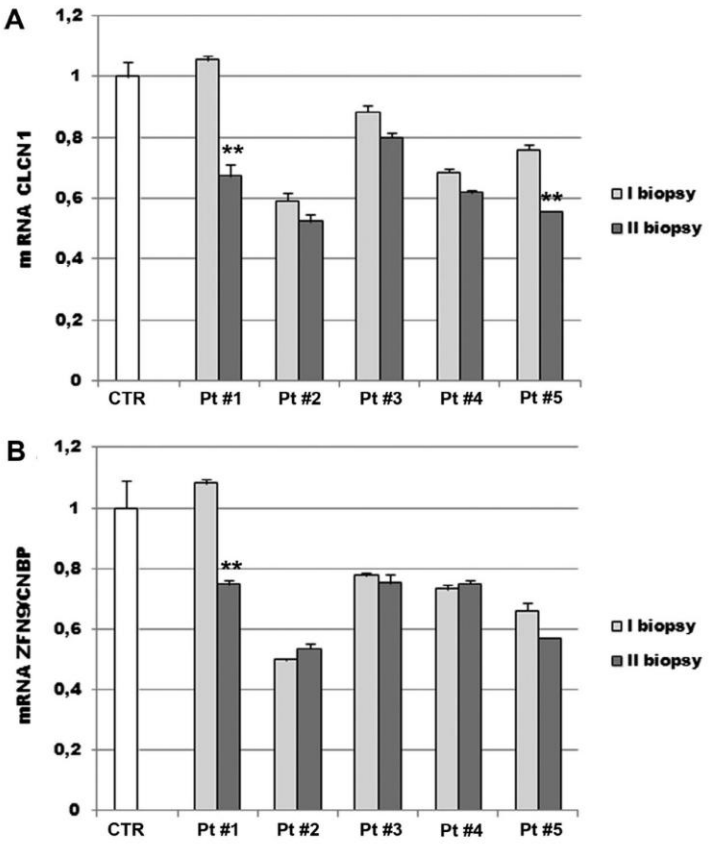

Fig. 6. Results of QRT-PCR experiments to quantify the expression level of the *CLCN1* (A) and *ZNF9*/*CNBP* (B) mRNA in muscle samples from 2 control subjects and from two successive biopsies of 5 DM2 patients. Each experiment has been performed in triplicate and the relative amount of the *CLCN1* transcripts has been determined using the β_2-microglobulin as endogenous control gene. Histograms represent mean ± SD. Student *t*-test: $^{**}p < 0.001$.

by other Authors [33,34]. Furthermore, it is noteworthy that in patients examined in our work, CUGBP1 expression level increases during aging. It has been reported that aging increases the amounts of CUGBP1 in the liver and adipose tissue [35,36] through the formation of a multi-protein complexes of CUGBP1 such as CUGBP1-eIF2 [37]. Interestingly, formation of an identical complex has been observed in skeletal muscle of patients with DM2 in which this complex up-regulates translation of proteins involved in muscle development [32]. Also, a reduction of CNBP levels may play a role in DM2 pathology and might explain phenotypic differences between DM1 and DM2 [34,38–40]. In this work we have observed a reduction of CNBP both at the mRNA and protein level in DM2 muscle, however this reduction is not related to a worsening of muscle histology.

Since DM2 multisystemic phenotype is associated to a spliceopathy of several genes [7,8], we have analyzed if the worsening of clinical symptoms may be related to an increase of the expression of the pathological isoforms of some genes involved in DM2 pathology. However, the analysis of alternative splicing of *IR*, *SERCA1*, *CLCN1*and *MBNL1* genes in two successive muscle biopsies does not evidence a worsening of alternative splicing alterations even after a 10-year interval between the two biopsies. Myotonia is the primary symptom in people who have DM and it gets worse over time [27]. It is well known that myotonia in DM patients is caused by the loss of CLCN1 mRNA and protein expression in DM1 skeletal muscle tissue due to aberrant splicing of the *ClCN1* pre-mRNA [41,42]. In patients considered in this work we have not observed a worsening of *CLCN1* splicing alteration though a decrease of CLCN1 mRNA has been observed in all patients. However we cannot assert if myotonia worsens over time in our DM2 cohort due to mexiletine treatment.

Taken together our data confirm that disease progression in DM2 is slow since histological and biomolecular alterations observed in skeletal muscle are minimal even after a 10-year interval. Moreover data indicate that muscle morphological alterations evolve more rapidly over time than the molecular changes thus indicating that muscle biopsy is a more sensitive tool than biomolecular markers to assess disease progression

at muscle level. These observations would need to be confirmed with a larger sample of muscle biopsies.

Understanding the molecular mechanisms that play a role in DM disease progression is important for identifying new therapeutic agents, when considering that in DM patients not only skeletal muscle is affected, but also the central nervous system and the heart.

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Premature senescence in primary muscle cultures of myotonic dystrophy type 2 is not associated with p16 induction

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Abstract

Myotonic dystrophy type 1 (DM1) and type 2 (DM2) are multisystemic disorders linked to two different genetic loci and characterized by several features including myotonia, muscle weakness and atrophy, cardiac dysfunctions, cataracts and insulin-resistance. In both forms, expanded nucleotide sequences cause the accumulation of mutant transcripts in the nucleus deregulating the activity of some RNA-binding proteins and providing an explanation for the multisystemic phenotype of DM patients. However this pathogenetic mechanism does not explain some histopathological features of DM skeletal muscle like muscle atrophy. It has been observed that DM muscle shares similarities with the ageing muscle, where the progressive muscle weakness and atrophy is accompanied by a lower regenerative capacity possibly due to the failure in satellite cells activation. The aim of our study is to investigate if DM2 satellite cell derived myoblasts exhibit a premature senescence as reported for DM1 and if alterations in their proliferation potential and differentiation capabilities might contribute to some of the histopathological features observed in DM2 muscles. Our results indicate that DM myoblasts have lower proliferative capability than control myoblasts and reach *in vitro* senescence earlier than controls. Differentely from DM1, the p16 pathway is not responsible for the premature growth arrest observed in DM2 myoblasts which stop dividing with telomeres shorter than controls. During *in vitro* senescence, a progressive decrease in fusion index is observable in both DM and control myotubes with no significant differences between groups. Moreover, myotubes obtained from senescent myoblasts appear to be smaller than those from young myoblasts. Taken together, our data indicate a possible role of DM2 premature myoblast senescence in skeletal muscle histopathological alterations *i.e.*, dystrophic changes and type 2 fibre atrophy.

Introduction

Myotonic dystrophies (DM) are autosomal dominant multisystemic disorders characterized by a variety of multisystemic features including myotonia, muscular dystrophy, cardiac dysfunctions, cataracts and insulin-resistance.[1] Myotonic dystrophy type 1 (DM1) is the most prevalent form of adult neuromuscular disorder and is caused by an expanded (CTG)n in the 3' untranslated region of the Dystrophia Myotonic Protein Kinase (*DMPK*) gene.[2-4] The second form of DM, myotonic dystrophy type 2 (DM2), is caused by the expansion of a tetranucleotidic repeat (CCTG)n in the intron 1 of the CCHC-type zinc finger, nucleic acid-binding protein (*CNBP*) gene.[5,6] In both forms, the mutant transcripts accumulate in nuclear foci altering the function of several alternative splicing regulators, which are necessary for the physiological processing of mRNAs.[7-10] These alterations lead to a spliceopathy *i.e.*, an aberrant alternative splicing of different genes that explain different features of the DM multisystemic phenotype.[11,12] However, at the skeletal muscle level, still there is no mechanistic explanation for the muscle weakness and atrophy observed in DM patients or for the muscle histopathological features characteristic of this disease which includes fibre atrophy-hypertrophy, increased number of central nuclei, and presence of fibres with nuclear clumps.[13-15]

It has been obseved that in DM muscle tissue there is decreased muscle regeneration in response to the ongoing muscle loss and dystrophy[1] thus it is possible that the blunted repair response may result from impaired myogenesis in adult DM muscle. Interestingly, DM muscle shares apparent similarities with the ageing muscle where the progressive muscle weakness and atrophy is accompanied by a slower regenerative capacity.[16-20] Satellite cells, the muscle precursor cells, provide the potential for both pre and post-natal growth of skeletal muscles, and for its regeneration following injury.[21,22] In normal muscles, satellite cells are quiescently located between the sarcolemma and the basal lamina of mature myofibres; following injury they become activated and then proliferate and fuse into myotubes to regenerate or repair muscle fibres.[23,24] It is known that the regenerative capacity of skeletal muscle depends on the number of progenitor cells which declines with age in humans and on their activation, proliferation and differentiation potential.[25-27] The proliferative potential of human satellite cells decreases during postnatal muscle growth due to the replicative senescence.[25,26] Replicative senescence may be caused by progressive telomere shortening at each cellular division or by additional pathways such as the p16 stress pathway, which could also interfere with the proliferation

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Key words: Myotonic dystrophy, aging, myoblasts, p16, telomeres.

Contributions: RC, study design, muscle histopathological evaluation, myoblast cultures, data analysis and interpretation, manuscript drafting; LVR, myoblast cultures, western blotting analysis, data analysis and interpretation, participation in manuscript drafting; AB, genetic analysis; data analysis and interpretation; manuscript revision; GR, telomere lenght analysis and interpretation of the data; BF, data analysis, clinical evaluation, manuscript revision; EC, data analysis, manuscript revision; GM, study conceptualization, muscle histopathological and clinical evaluation, manuscript revision, final approval of the version to be published.

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capacity of human myoblasts by inducing premature senescence.[28,29] It is therefore possible that an early senescence of satellite cells could affect both the regeneration process and the maintenance of muscle mass, since the differentiation program of senescent myogenic precursor cells would become defective due to impaired myogenesis and/or the down-regulation of the myogenic regulatory factors. Indeed, Bigot *et al*. observed that senescent myoblasts are still able to fuse and form myotubes, yet significantly smaller and with a significant reduction in the number of nuclei per myotube and in the fusion index, compared with those obtained from young myoblasts.[29] Such defects have been observed in diseases involving repeated cycles of degeneration-regeneration, such as the Duchenne muscular dystrophy (DMD), where satellite cells display a defective phenotype related to the exhaustion of proliferative capacity.[30] Defective myoblasts have also been observed from biopsies of patients suffering from other neuromuscular disorders such as oculo-pharyngeal muscular dystrophy, sporadic inclusion body myositis and DM1.[31-33] In a recent report, it has been demonstrated that satellite cells isolated from DM1 patients reached the proliferative senescence when their mean telomere lengths were longer than those observed in passage-matched control cells indicating that DM1 cells reach proliferative arrest prematurely, independently of telomere shortening.[34] A premature growth arrest of satellite cells from congenital DM1 patients carrying large CTG expansions has also been observed, and the p16 pathway appears to be responsible for this phenomenon.[35] As reported for adult DM1 myoblasts, these foetal myoblasts stop dividing when their telomeres are significantly longer than those of control cells.[35] The early occurrence of senescence-related features in satellite cell-derived myoblasts suggests that satellite cells from DM1 patients have a reduced regeneration capability, which would contribute to the muscular dystrophic phenotype.

Recently, our group has shown that DM2 myoblasts at early culture passages, when they proliferate at a similar rate as the controls, already show senescence related features mainly consisting in the early appearance of cytological alterations and impairment of the pre-mRNA maturation pathways.[36] The aim of our study is to elucidate if satellite cell derived myoblasts obtained from skeletal muscle of DM2 adult patients also exhibit a premature senescence as reported for DM1 and if alterations in their proliferation potential and differentiation capabilities might contribute to some of the clinical and histopathological features observed in DM2 muscles.

Materials and Methods

This study was authorized by the Institutional Ethics Committee of the Local Health Unit (ASL MI2, Melegnano, Milan, Italy) and was conducted according to the principles expressed in the Declaration of Helsinki, the institutional regulation and Italian laws and guidelines. Written informed consent were obtained from the patients for all blood samples and muscle biopsies used in this study.

Patients

Human muscle biopsies from *biceps brachii* muscle were taken under sterile conditions from DM1 (n=3) and DM2 (n=4) patients enrolled in Ilalian DM Registry and from age matched subjects with no sign of neuromuscular disease (n=4) used as controls. The diagnosis of DM was based upon the clinical diagnostic criteria set by the International Consortium for Myotonic Dystrophy.[37] DM2 diagnosis was performed by fluorescence in situ hybridization on muscle frozen sections using a $(CAGG)_5$ probe as previously reported by Cardani *et al*.[38] to verify the presence of ribonuclear inclusions. DM1 and DM2 genotyping were performed on genomic DNA extracted from peripheral blood leukocytes as previously described.[39-41]

Muscle histopathology and immunohistochemistry

Biceps brachii biopsies were fresh-frozen in isopentane cooled in liquid nitrogen. Histopathological analysis was performed on serial sections (8 µm) processed for routine histological and histochemical stainings. A standard myofibrillar ATPase staining protocol was used after preincubation at pH 4.3, 4.6, and 10.4.[42] Immunohistochemical staining was performed on serial sections (6 µm) air-dried and rehydrated in phosphate buffer pH 7.4 (PBS). Non-specific binding sites were blocked with normal goat serum (NGS; Dako, Glostrup, Denmark) at a dilution 1:20 in PBS containing 2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature. Sections were then incubated for 1 h at room temperature with mouse monoclonal primary antibodies against two different myosin heavy chain (MHC): MHCfast, diluited 1:400 in PBS+2%BSA (Sigma-Aldrich); MHCslow, diluited 1:400 in PBS+2%BSA (Sigma-Aldrich). After washing in PBS 3 times for 5 min (3x5 min), sections were incubated for 1 h at room temperature with goat anti-mouse biotinylated secondary antibody diluted 1:300 in PBS+2%BSA. After washing in PBS 3x5 min, sections were incubated for 30 min with Vectastain ABC complex (Vector Laboratories, Burlingame, CA, USA) and then with 3,3'-Diaminobenzidine (DAB) and hydrogen peroxide for 20 min. Nuclei were counterstained with Mayer's hematoxylin. Quantitative evaluation of fiber diameter was made as described previously by Vihola *et al*.[13] with Scion Image (Scion Corporation, Frederick, MD, USA) on images taken with a light microscope (1600x, original magnification). The size of muscle fibers was assessed by measuring the *smallest fiber diameter* and all data were elaborated using Microcal Origin (Microcal Software Inc., Northampton, MA, USA). The metahistograms were normalized to normal mean diameter for men and women. Relative atrophy and hypertrophy factors were also calculated.

Primary skeletal muscle cell cultures

The human satellite cells were isolated from muscle biopsies as reported by Cardani *et al*.[43] Myoblasts were grown in HAM's F10 medium (Sigma-Aldrich) supplemented with 15% FBS (Euroclone), 0.5 mg/mL albumin from bovine serum (BSA, Sigma-Aldrich), 0.5 mg/mL fetuin (Sigma-Aldrich), 0.39 µg/mL dexamethasone, 10 ng/mL epidermal growth factor, 0.05 mg/mL insulin, 3 mg/mL glucose, 100 U/mL penicillin and 100 µg/mL streptomycin (proliferative medium). For this study, cells from DM and control patients were plated at a density of 85,000 cells per 60 mm dishes or 50,000 cells per 35 mm dishes. Myogenic purity was evaluated by immunofluorescence using desmin as marker (see below). The percentage of desmin positive myoblasts was calculated as the number of positive cells *vs* the total number of cells observed. All starting cell populations used in this study had a myogenic purity higher than 80%. Moreover, the myogenic purity was monitored every 5 passages (7 days per passage for a total of 35 days) until senescence was reached. At least 100 cells were counted in at least 10 different optical fields randomly chosen.

Proliferative capacity and senescence

Cells from DM patients and controls were plated in triplicate at the density 50,000 per 35 mm dishes and, after 7 days, were trypsinized, counted with trypan blue and then replated at the same density (I passage; early stage). At each passage, every 7 days, the proliferative capacity was evaluated as mean population doubling (MPD) using the formula log (N/n)/log2 where N is the number of cells counted and n is the number of cells initially plated. Cultures were considered to be in senescence when cells have arrested their growth for 3 consecutive passages (late stage). As biomarker for replicative senescence, SA-β-

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gal, a β-galactosidase activity detectable in cultured cells undergoing replicative senescence but absent from proliferating cells, was evaluated according to Dimri *et al.*[44] on myoblast at early and late stages of proliferation. Quantification of SA-βGal positive cells was done by counting blue stained cells in 5 randomly selected fields and expressing the percentage of positive cells respective to the total number of cells visualized in each field.

Differentiative capacity

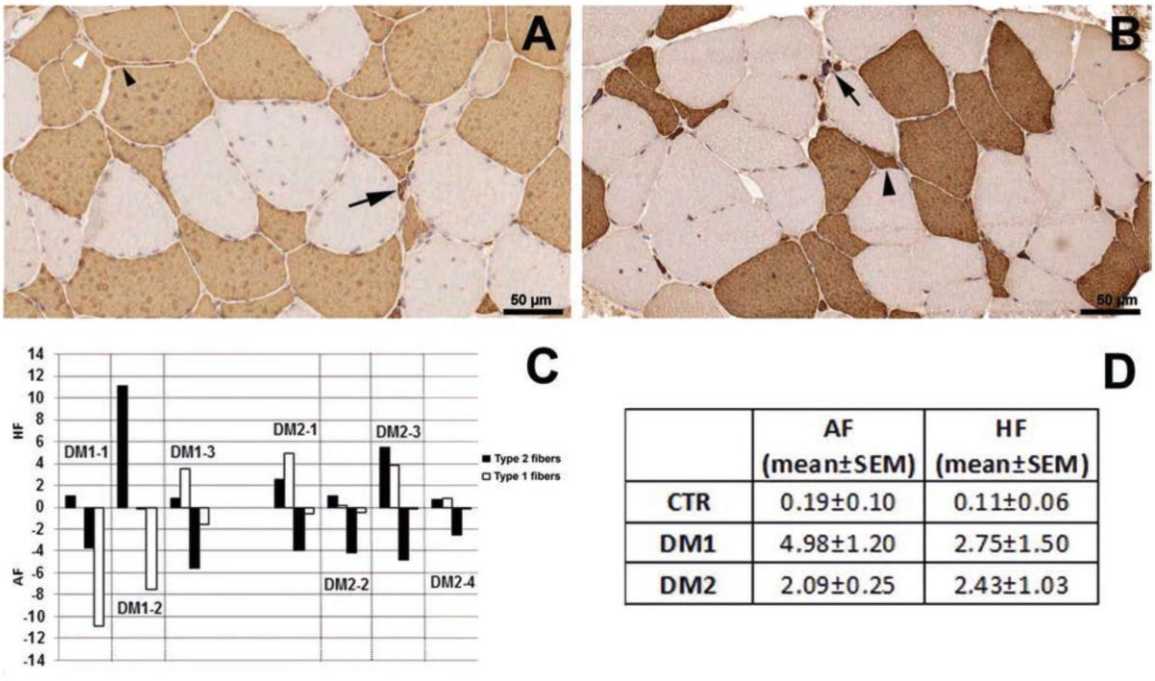
Myoblasts at early and late stages were plated at a high density in 35 mm dishes and, when they reached 80% of confluence, proliferative medium was replaced by differentiative medium (DMEM/High Glucose supplemented with 7% FBS, in presence of 100 U/mL penicillin, 100 μg/mL streptomycin and 0.01 mg/mL insulin) to allow myoblasts fusion. This condition of plating at high density prior to inducing differentiation was chosen in order to eliminate any bias in the quantification of fusion, which could be decreased by the poor migration of senescent cells. After 5 days in differentiative medium (T5), myotubes were analyzed in term of protein expression (MHC-fast myosin and myogenin, a myogenic factor involved in myoblast differentiation; see below) and fusion index to evaluate myoblasts differentiative capacity. Fusion index was determined as number of nuclei in multinucleated myotubes expressed as a percentage of the total number of nuclei. At least 100 nuclei were counted in at least 10 different optical fields randomly chosen.

Immunofluorescence

Immunofluorescence has been performed on proliferating myoblasts at early stage, on senescent myoblasts at late stage and on myotubes obtained from myoblasts at early and late stages. Myoblast and myotube (T5) cultures were fixed in 4% paraformaldehyde for 15 min at 4°C. After fixation, cells were washed several times in PBS and then permeabilized in 0.4% Triton X-100 in PBS for 5 min. After washing in PBS, non-specific binding sites were blocked with NGS (Dako-Cytomation) at a dilution 1:20 in PBS+2% BSA for 20 min at room temperature. Myoblast and myotubes were then incubated respectively with a primary antibody mouse monoclonal anti-desmin (CD33, Dako, 1:100 in PBS+2%BSA) and a mouse monoclonal anti MHC-fast (Sigma-Aldrich, 1:600 in PBS+2%BSA) for 1 h at room temperature. After washing in PBS 3x5 min, cells were incubated for 1 h at room temperature with secondary antibodies (goat anti mouse Alexa 488-labeled; Molecular Probes, Eugene, OR, USA; 1:400 in PBS+2%BSA). After washing in PBS 3x5 min, nuclei were stained with 165 nM 4,6-diamidino-2-phenylindole (DAPI). Cells were finally mounted with Mowiol and examined using a fluorescence microscope (LEICA FW4000).

Western blot analysis

During the *in vitro* ageing, the expression of different proteins involved in proliferation and/or in differentiation was analyzed by western blot (WB) on myoblasts or on myotubes (T5) protein extracts. WB analyses were performed on proliferating (early stage) and non-proliferating (late stage) myoblasts and on



	AF (mean±SEM)	HF (mean±SEM)
CTR	0.19±0.10	0.11±0.06
DM1	4.98±1.20	2.75±1.50
DM2	2.09±0.25	2.43±1.03

Figure 1. Cross-sections of skeletal muscle from DM1 (A) and DM2 (B) patient after immunostaining for fast myosin. Note the presence of type 1 (white arrowhead) and type 2 (black arrowhead) atrophic fibers in DM1 section (A), while in DM2 section (B) type 2 fibre atrophy (black arrowhead) is evident. Black arrows indicate fast positive nuclear clumps. Relative hypertrophy factor (HF) and atrophy factor (AF) of each DM1 and DM2 patients (C) and mean values ± standard error of the mean (SEM) of HF and AF for each patient group (D). The results are based on the morphometric analysis of sections immunostained for MHC fast or slow myosin.

myotubes obtained from myoblasts at early and late stage. Whole cell extracts were prepared by the addition to the plates of lysis buffer [50 mM Tris–HCl, pH 7.4 (TBS), 200 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM b-glycerophosphate, 10 mM sodium fluoride, 1 mM sodium vanadate, Protease inhibitor cocktail (Sigma Aldrich)] and rubbed with a cell scraper. After incubating on ice for 15 min, samples were centrifuged at 5700g for 15 min at 4°C, and supernatant was collected and stored at -80°C. Pellets were suspended in 50 mM TBS with 5% SDS and stored at -80°C. Protein concentration in each sample was determined by using BCA assay kit (Pierce, Rockford, IL, USA). Eight µg of protein for each sample was loaded per lane and electrophoresed on 8% or 15% sodium dodecyl sulfate-polyacrylamide gels, and than transferred to nitrocellulose membranes (Bio-Rad Laboratories). After blocking non specific sites in TBS containing 5% BSA for 30 min at 42°C, membranes were incubated overnight at 4°C with different primary antibodies diluted in TBS+5%BSA+0.1%Tween20 for mouse monoclonal antibodies, and in TBS+5%BSA+0.3%Tween20 for rabbit polyclonal antibodies. The expression of desmin (1:500; Clone D33; Dako-Cytomation), MyoD (1 µg/mL; Clone 5.2F; Sigma-Aldrich), p16 (1:200; Clone H-156; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and PCNA (1:5000; Clone PC10; Dako-Cytomation) was analyzed in myoblast cultures, while MHCf (1:5000; Sigma-Aldrich) and myogenin (1 µg/mL; Clone F12B; Sigma-Aldrich) were analyzed in myotubes cultures. After several washes in TBS+0.2%Tween20, membranes were incubated with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit secondary antibodies diluted respectively 1:5000 and 1:10,000 in TBS+5% BSA+0.2%Tween20 (Jackson Immuno Research Laboratories, West Grove, PA, USA). Membranes were washed and immune complexes were detected using the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were performed in triplicate.

DNA extraction and telomere length analysis

Genomic DNA has been extracted from $1x10^6$ DM1 (n=2), DM2 (n=4) and control (n=3) myoblasts at early and late stages with FlexiGene kit (QIAGEN, Hilden, Germany), following the manufacture's instructions. The quantification of telomere lengths has been assessed through Southern blot analysis of terminal restriction fragments (TRF) using the TAGGG Telomere Length Assay (Roche). Briefly, 2 µg of genomic DNA from each sample were digested with HinfI and RsaI enzymes, digested fragments were resolved by gel electrophoresis in a 0.8% agarose gel and transferred onto a nylon membrane. The blotted DNA fragments were hybridized to a digoxigenin (DIG)-labeled $(TTAGGG)_4$ probe and incubated with a anti DIG-specific antibody covalently coupled to alkaline-phosphate. Metabolized CDP-Star substrate is then detected by chemiluminescence indicating the location of the TRF on the blot. The average TRF length was determined by comparing the location of the TRF on the blot relative to a molecular weight standard. The TRF signal has been scanned and digitalized by STORM 860 (Molecular Dynamics, Sunnyvale, CA, USA) and ImageQuant software. Mean TRF lengths have been calculated for each sample on three independent gels, as reported previously by Kimura *et al.*[45] The number of cell division being known, the shortening velocity of telomeres has been determined, and it has been calculated whether in DM cells the telomere loss is higher than in controls.

Statistical analysis

To determine significance between two groups, comparisons were made using an unpaired Student's *t*-test. The groups were considered statistically different at $P<0.05$.

Results

Patients and skeletal muscle histopathology

This study was performed on DM1 and DM2 age-matched patients compared to subjects with no sign of neuromuscular disease used as controls. Although the number of patients analyzed in this study was small, DM1 and DM2 groups were homogeneous: the DM1 cohort was represented by 3 classical adult patients mildly affected with a range of CTG repeat expansion of 361.6±102.8 (E2) and the MIRS was 3-4, the DM2 cohort was represented by 4 patients with classical PROMM phenotype and fluctuating myotonia, except for patient #3 (Table 1).

Analysis of muscle sections immunostained for MHC fast or slow myosin allows us to detect and measure fibers smaller than 5 µm, including all nuclear clump fibers which are recognizable by the presence of a thin rim of immunoreaction around the nuclei (Figure 1 A,B). All patients used in this study show an increase of fibre atrophy. In particular, an increase of both type 1 and type 2 fiber atrophy is present in DM1 patients and a prevalent type 2 fibre atrophy is evident in DM2 patients.

Table 1. Clinical data of patients analyzed in this study.

Patient	Sex	Age at onset	Age at biopsy	CTG expansion	Myotonia	MIRS°	MRC#
CTR-1	F	-	43	-	Not present	-	-
CTR-2	M	-	44	-	Not present	-	-
CTR-3	M	-	48	-	Not present	-	-
CTR-4	F	-	48	-	Not present	-	-
DM1-1	M	38	39	370	±	3	128.35
DM1-2	F	35	45	460	++	4	128.35
DM1-3	M	39	47	255	+	4	105.36
DM2-1	F	35	51	-	+	-	118.70
DM2-2	F	35	53	-	±	-	132.99
DM2-3	F	19	45	-	++	-	128.68
DM2-4	F	54	59	-	+	-	109.68

°Muscle Impairment Rating Scale, stage of the disease for myotonic dystrophy type 1 (DM1) patients.[53] #Medical Research Council, scale for muscle strength; scale (0-5 grade) on 15 muscles at both sides in the upper and lower limbs for a total of 150 maximum score. CTR, control; DM1, myotonic dystrophy type 1; DM2, myotonic dystrophy type 2; F, female; M, male.

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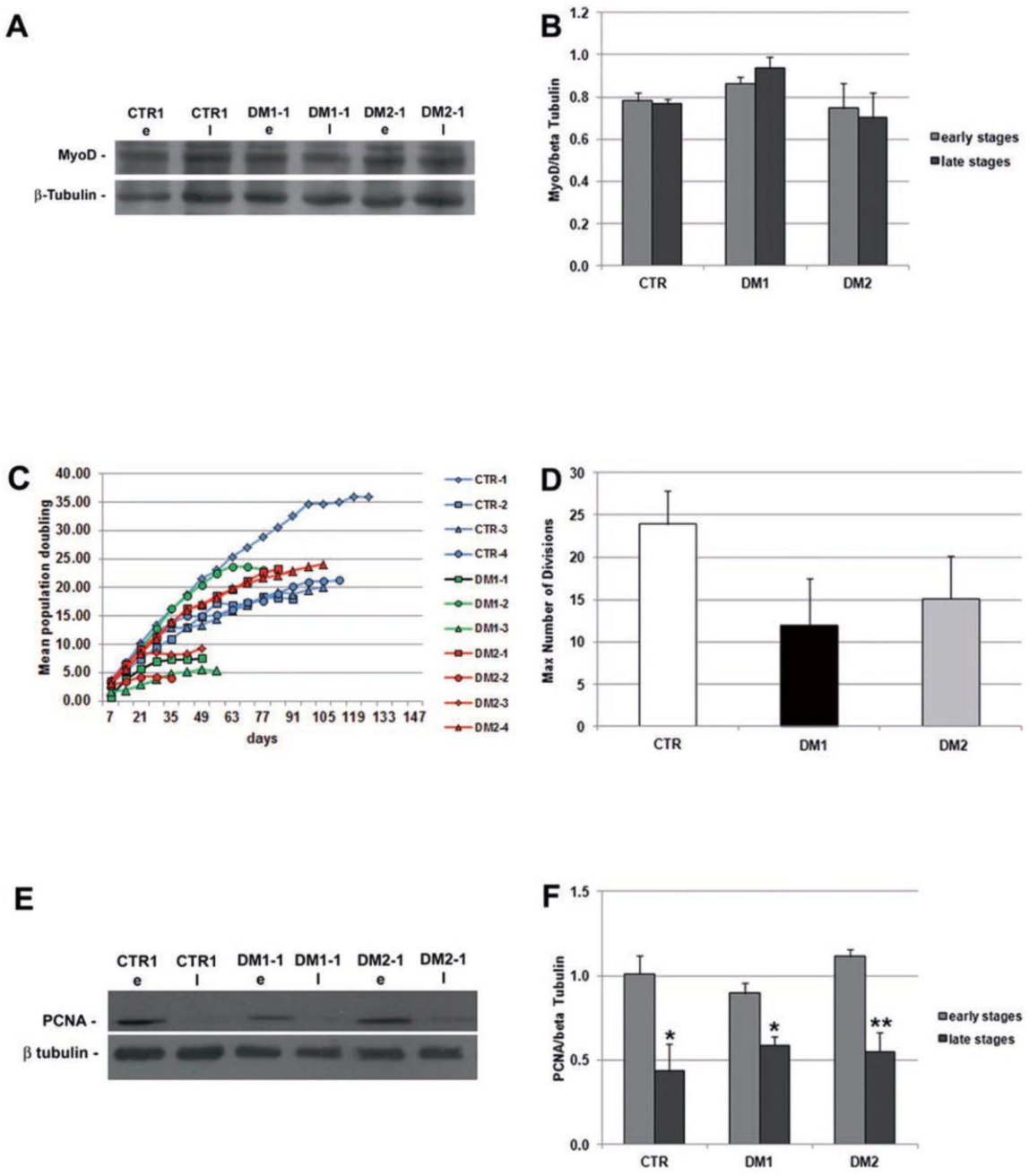

Figure 2. A) Representative western blot analysis of MyoD expression in myoblasts at early (e) and late (l) passages of proliferative lifespan obtained from *biceps brachii* muscle samples from healthy (CTR), DM1 and DM2 patients; density of the bands has been normalized to β-tubulin expression used as internal control. B) Histograms representing mean values of MyoD protein expression analysed by densitometry of CTR (n=4), DM1 (n=3) and DM2 (n=4) patients; scale bars are for SEM. MyoD expression is similar in young and senescent cells indicating that the myogenic purity persists to be high throughout the experiment. C) Lifespan plots of control and DM myoblasts. D) Mean of max number of divisions of CTR (n=4), DM1 (n=3) and DM2 (n=4) patients; scale bars represent SEM; the average proliferative lifespan of the DM myoblasts is reduced as compared to that of control cells. E) Representative western blot analyses of PCNA in the early (e) and late (l) stages of myoblast proliferative lifespan; the results have been normalized to the expression of β-tubulin. F) Histograms representing mean values of PCNA protein expression analyzed by densitometry of CTR (n=4), DM1 (n=3) and DM2 (n=4) patients; scale bars represent SEM; levels of PCNA are significantly decreased in both DM and control senescent cells compared to young cells. *P<0.05, **P<0.01.

Hypertrophic type 1 and type 2 fibers were also present in DM patients (Figure 1C). The evaluation of atrophy (AF) and hypertrophy (HF) factors for each patient group are reported in Figure 1D.

Myoblast cultures and proliferative capacity

The myogenic purity of cell preparations was evaluated from the percentage of cells expressing desmin. Immunofluorescence assay revealed that the cell preparations used in this study were quite similar in terms of the desmin expression. The myogenic purity was around 75-80% throughout the experimental procedures (*data not shown*). Also MyoD protein expression indicated that the myogenic purity remained similar in the early and late stages both in DM and control cultures (Figure 2 A,B).

The *in vitro* proliferative capacity of myoblasts obtained from skeletal muscle biopsies of 3 DM1 and 4 DM2 patients was compared to that of myoblasts obtained from 4 age-matched unaffected individuals used as controls. As shown in Figure 2C, an interindividual variability was evident in both DM1 and DM2 patient group, however the average proliferative lifespan of the DM1 and DM2 myoblasts was reduced by 50% and 36% respectively as compared to that observed in control cells. Moreover, DM1 (9 passages) and DM2 (10 passages) myoblasts stopped dividing earlier compared with control cultures (15 passages) (Figure 2C). Myoblasts from DM1 and DM2 patients showed a clear reduction of the maximum number of divisions compared to those observed in controls cells (Figure 2D). Analysis of PCNA expression showed a significant reduction of protein expression in the late passages compared to the early stages both in DM and control cells (Figure 2E,F). The expression of PCNA in the late passages indicated that the majority of DM and control cells were arrested in G1 phase of the cell cycle.

Senescence analysis

To evaluate if a mechanism similar to senescence was responsible for the early proliferative growth arrest of DM myoblasts, we analyzed biomarkers usually observed in senescent cells. At early passages DM and control cells were actively dividing with rare or no senescent cells being detected but as the cells reached their late passage, the number of senescent cells positive for SA-β gal increased dramatically in both DM and control cells (Figure 3). Moreover, DM and control myoblasts at the late stages of proliferation showed a flattened morphology with an enlarged cytoplasm and extended cytosolic processes, a morphology reminiscent of that observed when myoblasts reach a senescent state (Figure 4).

We examined the expression of p16 protein at the beginning and at the end of DM lifespan to verify if this mechanism was also involved in the premature proliferative arrest observed in DM2 myoblasts *in vitro*. Our analysis indicate that p16 was overexpressed in DM1 cells both at the early and late stages compared to controls, while p16 protein expression appeared to be similar in DM2 and control myoblasts at both stages analyzed (Figure 5 A,B). Critically short telomeres trigger loss of cell viability in tissues, which has been related to alteration of tissue function and loss of regeneration tissue capabilities. Since it has been observed that DM1 myoblasts stopped dividing prematurely independently of telomere shortening,[34,35] we have analyzed telomere loss induced by *in vitro* aging in satellite-cell-derived myoblasts, isolated from muscles of DM1 (n=2), DM2 (n=4) patients and from age-matched control subjects (n=3). As shown in Figure 5C, satellite cells isolated from DM1 patients stopped growing prematurely with telomeres longer than controls (respectively 11.4±1.1 Kb and 9.5±0.3 Kb), differently from what observed in DM2 myoblasts which stop dividing with a median telomere length of 8.3±1.7 Kb even if a wide variability in mean TRF length can be observed in DM samples. It should be noted that the two DM2 cultures (#2 and #3, Figure 2C) showing the most premature growth arrest also showed the shortest telomere length at the early stages probably indicating an excessive proliferation *in vivo* before their isolation (*data not shown*). Moreover these two DM2 patients also show the highest values of atrophy factor *in vivo* (Figure 1C). As shown in Figure 5D, DM2 satellite cells has a clear two-fold decrease in the amount of telomeric DNA at every cell passage compared to controls whereas in DM1 cells the mean bp loss per division is 30% lower than controls.

Differentiative capacity

Myoblasts at early and late stages of proliferation were induced to fuse into myotubes during 5 days of differentiation (T5) in order to evaluate their differentiative capacity. Although the senescent cells were still able to form myotubes, they appeared to be significantly smaller than those formed by the young cells, which also formed branched myotubes not observable in senescent cultures (Figure 6 A-F). As observed in Figure 6G, a significant reduction in fusion index is observable both in DM and control myoblasts which has reached a proliferative arrest compared to proliferating myoblasts. This reduction appears to be more evident in senescent myoblasts from DM1 patients who also show higher value of atrophy factor compared to DM2. Moreover, the expression of myogenin appeared to be decreased both in DM and control myotubes obtained from non-proliferating myoblasts as compared to those obtained from proliferating myoblasts (Figure 6 H,I).

Discussion

Many symptoms of adult form of DM1 and DM2, such as muscle weakness and wasting, cataracts, and cardiac arrhythmias, are reminiscent to normal aging. Recent data on dystrophic skeletal muscle myonuclei have demonstrated alterations of mRNA pathways similar to those observed during aging.[19,46-48] Increasing evidence has demonstrated that in age-related myopathies, such as sarcopenia and myotonic dystrophy, the progressive muscle weakness and atrophy are characterized by impaired muscle regeneration due to satellite cells premature senescence that limits their proliferative potential.[1,35,49,50] In DM1 muscles the number of satellite cells is increased compared to muscles from non-affected individuals; however, DM1 cells do not seem to be able to counteract the progressive muscle atrophy due to a reduced proliferative capacity triggered by a mechanism of premature growth arrest.[34]

It is known that primary DM myoblasts do not show evident morphological abnormalities and are capable of normally differentiating;[43,51,52] however, we have recently observed that DM2 myoblasts are characterized by senescence related features mainly consisting in the early appearance of cytological alterations and impairment of the pre-mRNA maturation pathways.[36] To further investigate DM2 myoblasts aging, we have analyzed if cultured myoblasts from DM2 patients differ from myoblasts of age-matched DM1 and normal individuals in terms of cell proliferation, morphology, differentiation and senescence during *in vitro* aging and if alterations in their proliferation potential and differentiation capabilities might contribute to some of the clinical and histopathological features observed in DM2 muscles.

Our results seem to indicate that DM1 and DM2 myoblasts are characterized by a premature proliferative growth arrest compared to healthy myoblasts. Moreover, a mechanism similar to senescence appear is to be responsible for the early proliferative growth arrest because DM cells expressed biomarkers usually observed in senescent cells much earlier in their replicative lifespan as compared with age-matched control cultures. These data suggest that the *in vivo* regenerative capacity of DM satellite cells might be constitutively impaired. Furthermore, while the p16 pathway

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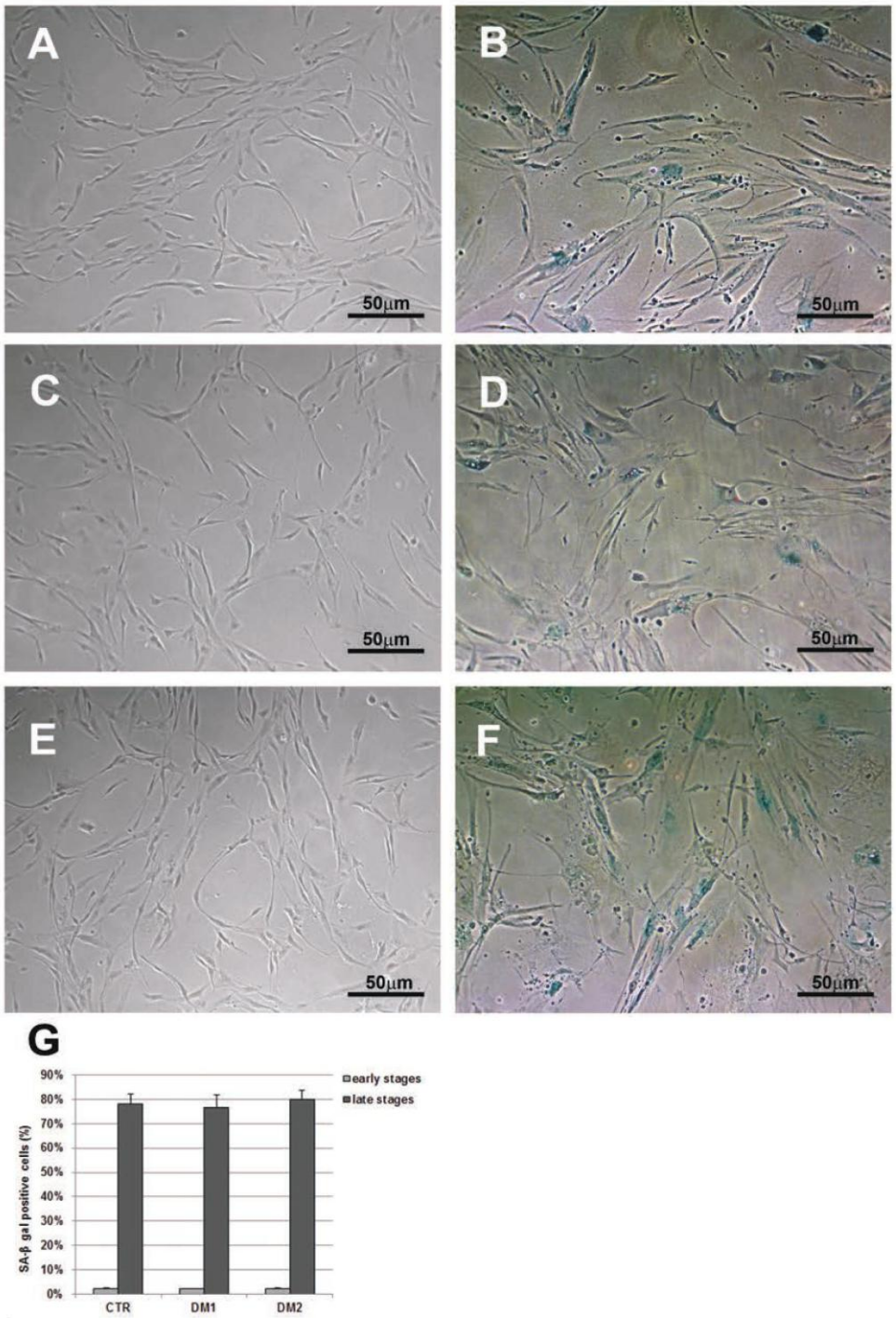

Figure 3. Representative images of CTR (A,B), DM1 (C,D) and DM2 (E,F) myoblasts stained for the senescence-associated β-galactosidase (SA-βGal) activity. At the proliferative (early) stages, very few cells were positive (A,C,E). At the high cell passage (late stages), myoblasts appear large, with the characteristic cytoplasmic blue staining typical of senescence (B,D,F). G) Quantification of the amount of SA-βGal positive cells shows a dramatic increase in the percentage of positive cells at the late stages; scale bars represent SEM.

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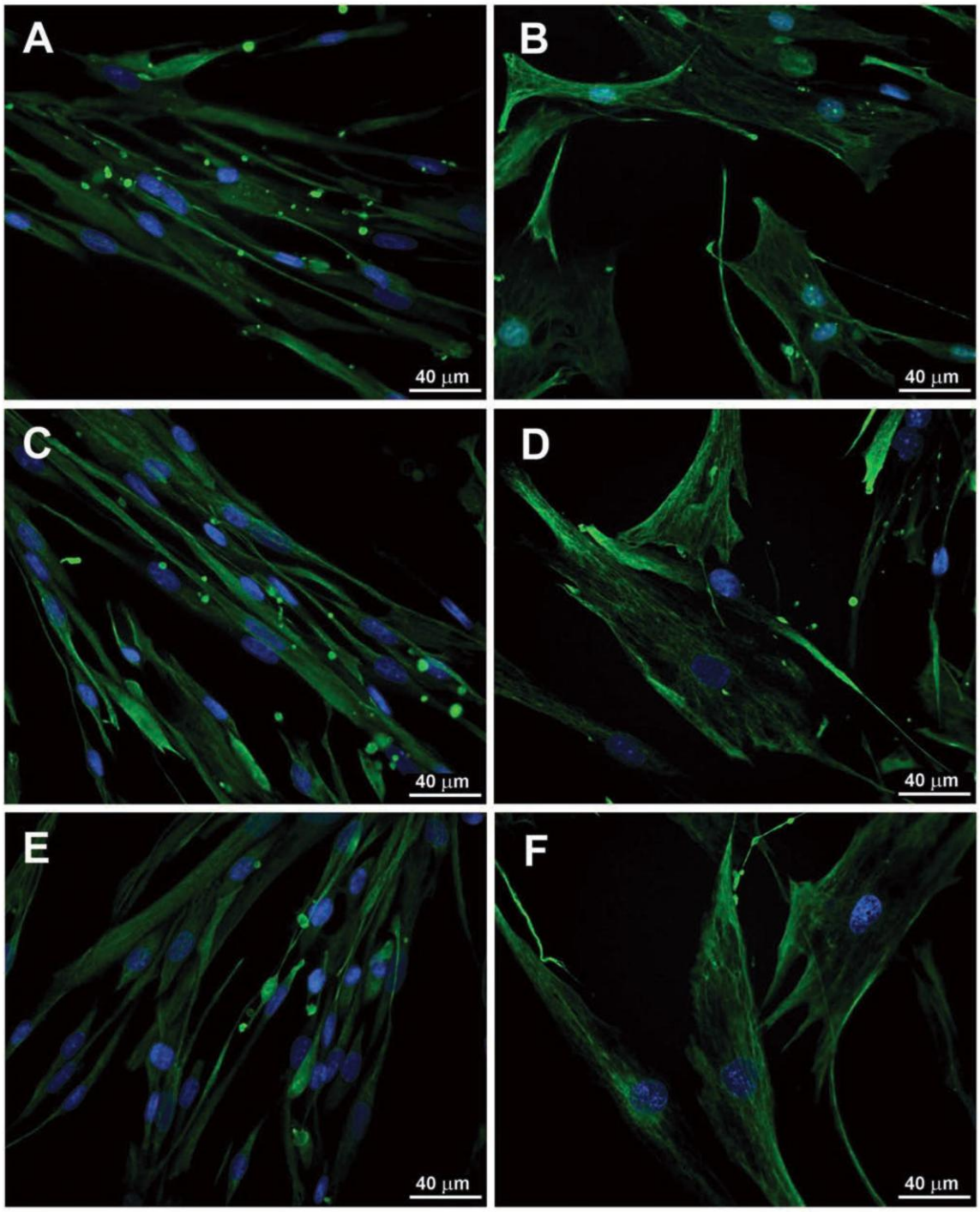

Figure 4. Representative images of CTR (A,B), DM1 (C,D) and DM2 (E,F) myoblasts immunostained for the myogenic marker, desmin. Both DM and control myoblasts at early stages of proliferation were relatively small and elongated (A,C,E), while at the late stages of proliferation they showed a flattened morphology with enlarged cytoplasm and extended cytosolic processes (B,D,F). Nuclei have been visualized with DAPI (blue).

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appear to be responsible for the premature growth arrest in DM1 probably in response to a CTG-related stress,[35] our results suggest that this mechanism is not responsible for the proliferative arrest observed in DM2 myoblasts. On the contrary, differently from what observed in DM1 myoblasts and reported in literature,[33-35] DM2 myoblasts stop dividing with telomeres shorter than controls suggesting that the signaling involved in premature senescence depend on a telomere-driven pathway. CCTG expansion might interfere with the telomere homeostasis in DM2 cells, yet the mechanism causing an accelerated telomere shortening has to be clarified. Critically short telomeres trigger loss of cell viability in tissues, which has been related to alteration of tissue function and loss of regeneration tissue capabilities. Each time a satellite cell divides, its DNA loses a small portion of this telomeric sequence;[53] once a critical length is reached, the cell becomes senescent and can no longer play a role in regeneration. Telomere length is an important predictive biomarker indicative for the regenerative capacity of human satellite cells as shown in Duchenne muscular dystrophy in which a telomere shortening after continuous cycles of degeneration and regeneration has been reported.[30] Hence, it appears that CTG and CCTG expansions trigger *in vitro* a mechanism of myoblast premature senescence through two different pathways, which could explain the different histological alterations observed between DM1 and DM2 skeletal muscle as for example the selective type 2 fibre atrophy present in DM2 muscle. However, further studies will be necessary to better understand how different senescence mechanisms involved in DM1 and DM2 myoblasts could lead to the selective involvement of type 1 or type 2 fibers in DM1 and DM2 respectively. As previously reported,[29,54] our results indicate that replicative senescence deregulates the myogenic programme resulting in impaired myogenesis. A decrease of the expression of myogenin, a myogenic factor involved in myoblast differentiation, is observable in myotubes obtained from senescent myoblasts as compared to those observed from proliferating myoblasts. Moreover, even though the senescent myoblasts are still able to fuse, a significant reduction in the fusion index has been observed when compared with young cells. It should be noted that the reduction in fusion index appear to be more evident in senescent myoblasts obtained from DM1 patients indicating that myoblasts deficiency could be responsible of the more severe muscle histopathology observed in DM1 compared to DM2. The reduced differentiating capacity is due to defect in differentiation and/or fusion properties of the senescent myoblasts and not to a decrease in the motility of senescent cells, since differentiation in senescent cultures was induced in myoblasts seeded at very high density. Thus, the histopathological defects observed in DM muscle such as fibre atrophy and nuclear clumps, could be due at least in part to the inability of premature aged myoblasts to generate myotubes able to produce mature skeletal muscle fibers or to fuse with existing fibers and prevent them from

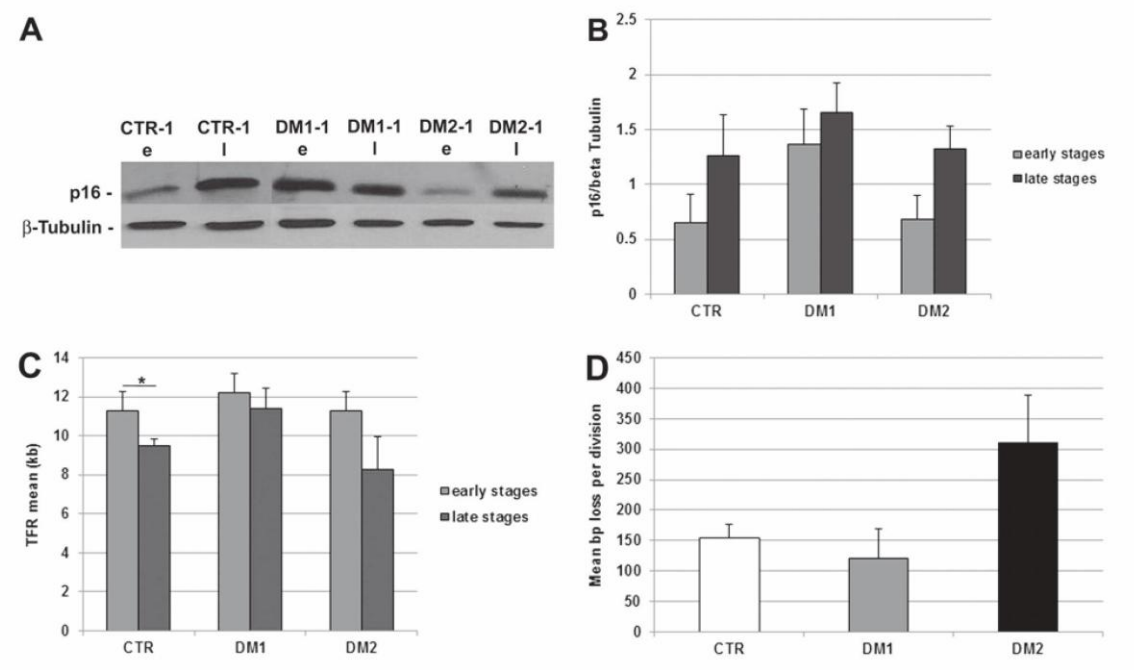

Figure 5. DM premature senescence pathway. A) Representative western blot analyses of p16 in the early (e) and late (l) stages of myoblast proliferative lifespan; the results have been normalized to the expression of β-tubulin. B) Histograms representing mean values of p16 protein expression analysed by densitometry in healthy (CTR; n=4), DM1 (n=3) and DM2 (n=4) patients; at both stages analyzed, p16 was more expressed in DM1 cells as compared to the controls while the expression was similar in DM2 and control myoblasts; scale bars represent SEM. C) Mean length of telomeric restriction fragments (TRF) measured on DM2 (n=4) and control (n=4) myoblasts at proliferative and senescent stage; senescent DM2 cells had shorter telomeres than the proliferating cells (*P<0.05); scale bars represent SEM. D) Mean length (in bps) of telomeric DNA lost per division in control and DM2 myoblasts; DM2 cells lost more bps per division than control cells; scale bars represent SEM.

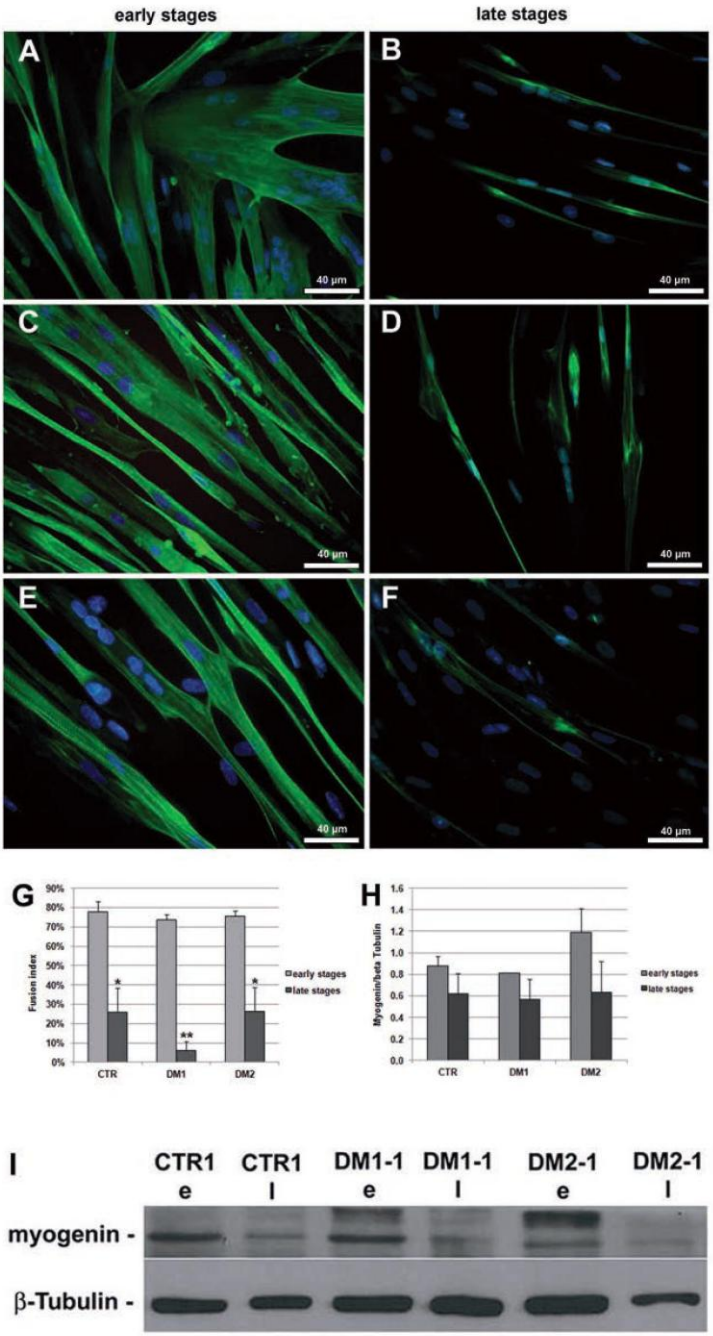

Figure 6. Differentiation capability of young and senescent myoblasts. A-F) Fast myosin immunofluorescence (green) of control (A,B), DM1 (C,D) and DM2 (E,F) myotubes from myoblasts at early and late stages of their proliferative lifespan, after 5 days of differentiation (T5). Nuclei have been visualized with DAPI (blue). Young myoblasts generate well-differentiated and cross-striated (C,E) myotubes larger than those formed by the senescent cells. G) Fusion index, i.e. the number of nuclei incorporated into myotubes as a percentage of the total number of nuclei, has been calculated in young and senescent myoblasts after 5 days of differentiation; a significant reduction of fusion index is observable in both DM and control myotubes obtained from senescent cells (*P<0.05, **P<0.01). H) Histograms represents mean values of myogenin protein expression analysed by densitometry of control (n=4), DM1 (n=3) and DM2 (n=4) patients; a reduced myogenin expression is observed in myotubes from senescent cells as compared to those obtained from young cells; scale bars represent SEM. I) Representative western blot analyses of myogenin in myotubes obtained from myoblasts at the early (e) and late (l) stages of their proliferative lifespan; the results have been normalized to the expression of β-tubulin.

atrophy. Although this study has been carried out on a limited number of cases, it seems indicate that, as in DM1 patients, myoblast premature senescence could explain the skeletal muscle wasting observed in patients affected by DM2.

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Part III

Post insulin receptor signalling abnormalities in myotonic dystrophy skeletal muscle cells

1. Background and significance of the project

Myotonic dystrophy (DM) is an autosomal dominant multisystemic disorder characterized by a variety of multisystemic features including myotonia, muscular dystrophy, cardiac dysfunctions, cerebral involvement, cataracts and insulin-resistance (Mankodi and Thornton, 2002; Meola and Moxley, 2004). Myotonic dystrophy type 1 (DM1) is caused by an expanded (CTG)n in the 3' untranslated region of the Dystrophia Myotonic Protein Kinase (*DMPK*) gene, while DM2 is caused by the expansion of a tetranucleotidic repeat (CCTG)n in the intron 1 of the CCHC-type zinc finger, nucleic acid-binding protein (CNBP) gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992; Ricker et al., 1994; Liquori et al., 2001). In both forms, the mutant transcripts accumulate in nuclear foci altering the function of alternative splicing regulators, CUGBP1 and MBNL1, which are necessary for the physiological processing of mRNAs. These alterations lead to aberrant alternative splicing of different genes (spliceopathy) that may explain different DM features (Taneja et al., 1995; Philips et al., 1998; Michalowski et al., 1999; Mankodi et al., 2001; Timchenko et al., 2001; Ranum and Day, 2004; Day and Ranum, 2005). DMs are characterized by metabolic dysfunctions such as insulin resistance, hyperinsulinemia, hypertriglyceridemia, increased fat mass, and a fourfold higher risk of developing Diabetes Mellitus type 2 (T2DM) (Meola 2000). However, in literature there are few studies aimed to clearly define the mechanisms underlying insulin resistance in DM. Savkur et al. (2001) suggested that splicing alteration of insulin receptor (IR) may play a role in peripheral insulin resistance. This splicing alteration leads to a higher expression of the fetal isoform A (IR-A, lacking exon 11) than the isoform B (Savkur et al., 2001). IR-B differs from IR-A by the inclusion of exon 11 that leads to a higher insulin signalling capability, potentially explaining DM insulin resistance (Savkur et al., 2001; 2004; Cardani et al., 2009; Loro et al., 2010). However, it cannot be excluded that post receptor signalling abnormalities could also contribute to the insulin resistance observed in DM patients and that insulin response defects might play a key role in the metabolic manifestations of DM, potentially leading to type 2 diabetes and abnormal muscle protein metabolism (Moxley et al., 1984; 1986).

For all these reasons, the aim of this project was to investigate the molecular mechanisms that induce insulin resistance in skeletal muscle cells obtained from both DM1 and DM2 patients.

2. Results and Discussion

This work has been performed on primary cultures of satellite-cell-derived myoblasts from biceps brachii biopsies of 3 adult DM1, 3 DM2 and 3 age-matched control patients. As reported by

Cardani et al. (2009), human muscle biopsy has been trimmed of blood vessels, fat and connective tissues and rinsed in phosphate-buffered saline, pH 7.4. The trimmed biopsies has been minced into pieces of 1 mm^3, dissociated with trypsin for 45 min at 37°C and isolated muscle cells have been plated in proliferative medium. As reported in figure 1, myoblasts obtained from satellite cells have been grown in proliferative medium without insulin until 80% confluence (T0), then the proliferative medium has been replaced with a differentiative medium containing a lower concentration of serum (15% FBS in proliferative medium *vs* 7% FBS in differentiative medium) to allow the fusion and differentiation of myoblasts into myotubes. After 5 days of differentiation (T5) human myotubes have been starved from serum overnight and then treated with insulin to study the activation of the insulin pathway in DM muscle cells.

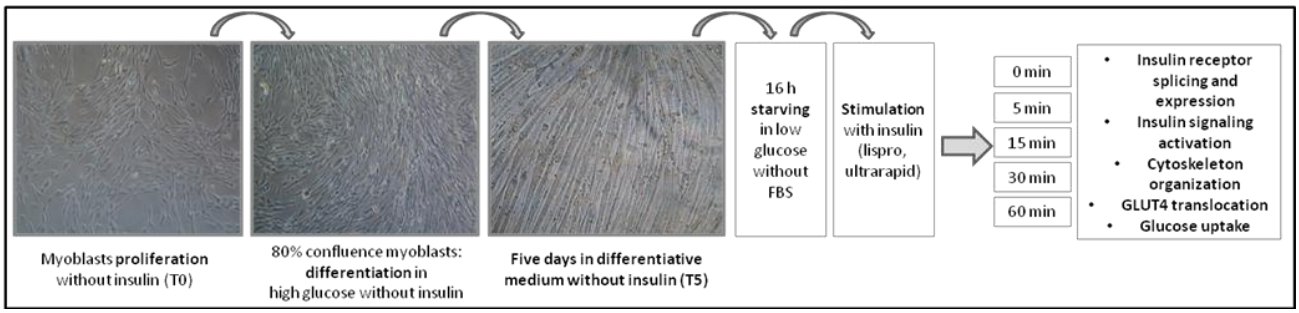

Figure 1: Schematic representation of the protocol used for *in vitro* study of the insulin pathway.

2.1 Insulin receptor splicing and expression

In this work we have analyzed insulin receptor (IR) expression and its alternative splicing in 5 days differentiated myotubes of healthy (control), DM1 and DM2 subjects (figure 2). The insulin receptor is a glycoprotein comprised of two α-subunits and two β-subunits linked by disulfide bonds (Whitehead et al., 2000). The binding of insulin to the α-subunit causes phosphorylation of the β-subunit on multiple tyrosine residues. In DM the nuclear accumulation of CUG/CCUG-containing RNA alters the levels of splicing factors which are required for alternative splicing of IR leading to an increase in IR-A:IR-B ratio in skeletal muscle biopsies and muscle cells of DM patients (Savkur et al., 2001; 2004; Cardani et al., 2009; Loro et al., 2010). However, the expression of insulin receptor (IR) in DM patients is still controversial since both normal and diminished RNA and protein levels have been reported (Savkur et al., 2001; Moxley et al., 1981; Morrone et al., 1997; Furling et al., 1999). Alternative splicing of the insulin receptor has been analyzed by RT-PCR and our results have shown that at five days of differentiation (T5) both control and DM myotubes express more fetal than adult IR isoform (56,8% IR-A/Total expression in CTR *vs* 59,1% IR-

A/Total expression in DM1 *vs* 56,6% IR-A/Total expression in DM2) (figure 2A, 2B). These results are consistent with those previously reported by Cardani et al. (2009) on IR alternative splicing regulation during human control myogenesis. Indeed, these authors reported that in control myoblasts (T0) and myotubes at early stages of differentiation (T4) the alternative splicing of the IR leads to a higher expression of the fetal isoform similar to what observed in DM muscle cells. Instead, at late stages of differentiation (T10), control but not DM myotubes express higher levels of adult IR-B isoform. QRT-PCR analysis have shown that there are no statistically significant differences in *IR* gene expression between CTR, DM1 and DM2 myotubes (figure 2C).

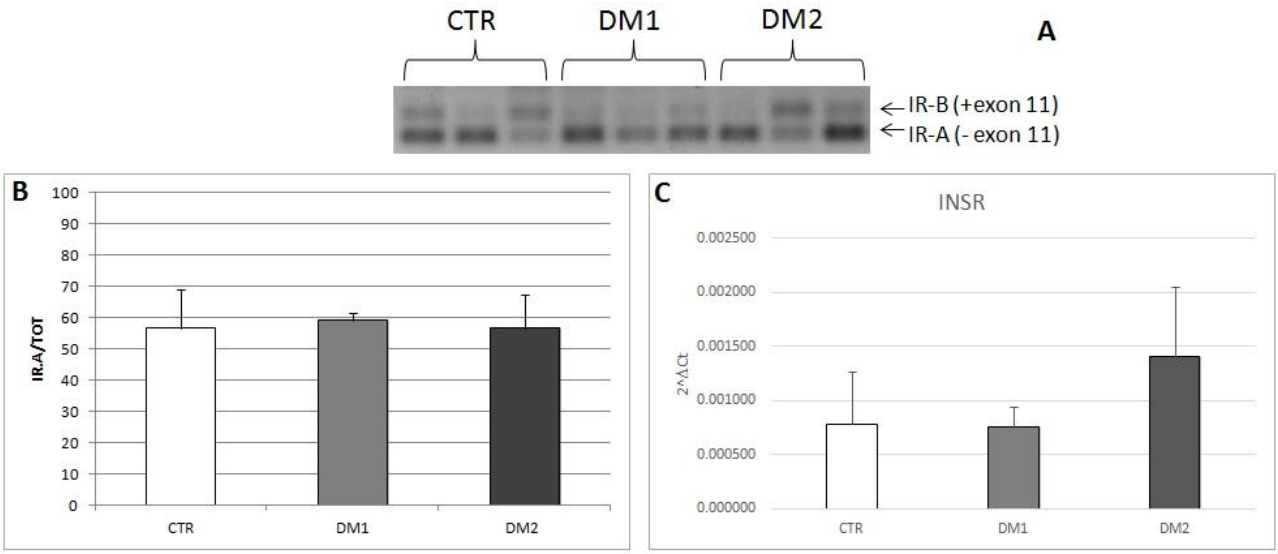

Figure 2: Insulin receptor (IR) alternative splicing and expression in 5 days (T5) differentiated human myotubes. **A)** Splicing products obtained by RT-PCR amplification of RNA isolated from myotubes obtained from control, DM1 and DM2 patients. **B)** Densitometric analysis measuring the fraction of aberrant gene isoform IR-A in control, DM1 and DM2 myotubes. Bars represent standard deviation. **C)** IR mRNA expression in T5 myotubes from control, DM1 and DM2 patients. The relative amount of IR transcripts has been determined using the GAPDH as endogenous control genes. Bars represent standard deviation.

2.2 Glucose uptake

Resistance to insulin action in human myotubes was first investigated at glucose uptake level. Glucose uptake is an important biological process for studying glucose metabolism. Among many different methods available for measuring glucose uptake, 2-deoxyglucose (2-DG) has been widely used because of its structural similarity to glucose. As with glucose, 2-DG can be taken up by glucose transporters and metabolized to 2-DG-6-phosphate (2-DG6P). 2-DG6P, however, cannot be further metabolized, and thus accumulates in the cells. The accumulated 2-DG6P is directly proportional to 2-DG (or glucose) uptake by cells. In BioVision's glucose uptake colorimetric assay kit, the 2-DG6P is oxidized to generate NADPH, which can be determined by an enzymatic

recycling amplification reaction. Figure 3A shows the insulin dose-response curve of 2-DG uptake in 5 days differentiated myotubes derived from 2 control subjects. Our results have confirmed that human skeletal muscle cultures provide a powerful tool for the investigation of the biochemical and genetic basis of peripheral insulin resistance, since glucose uptake remain responsive to insulin in cultured muscle cells from control subjects. In our control samples, each insulin concentration used induced an increase in glucose uptake. However, this increase resulted higher with 100 nM and 10 nM insulin (1,5 fold change with 1 μM *vs* 3,7 fold change with 100 nM *vs* 4,9 fold change with 10 nM). Since control myotubes exhibited the maximal stimulation at 10 nM insulin, we decided to perform the analysis of insulin resistance in DM muscle cells at this insulin concentration. The results of the glucose uptake assay on DM1 and DM2 myotubes have shown that DM muscle cells exhibit a lower glucose uptake after 10 nM insulin stimulation as compared to control cells. Interestingly, in DM myotubes the levels of glucose uptake after insulin stimulation were similar to those observed in myotubes before insulin stimulation (basal level), suggesting that the insulin pathway could be altered in DM muscle cells (figure 3B). These results are consistent with the results observed by Furling et al. (1999) in CDM (the congenital phenotype of myotonic dystrophy type 1) myotubes, where the treatment of CDM muscle cells with 10 nM insulin had no significant effect on glucose uptake.

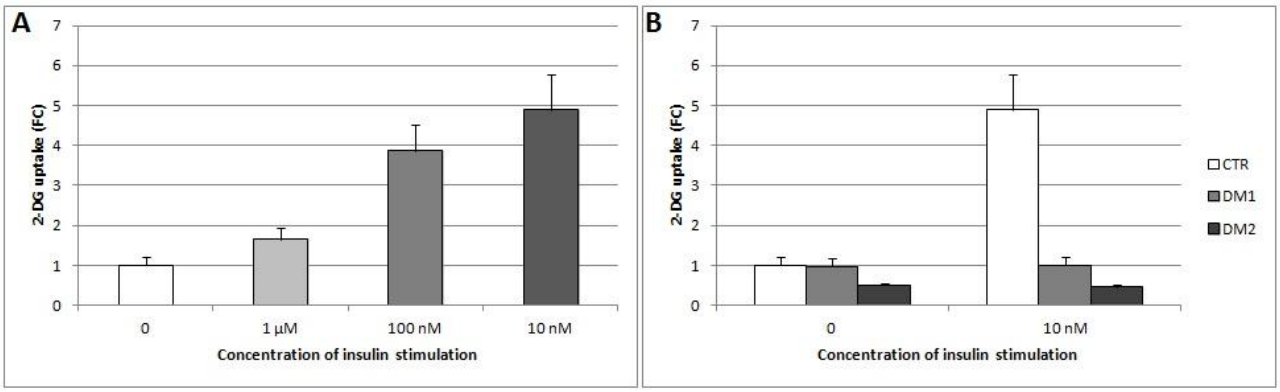

Figure 3: A dose-response histogram of insulin action on 2-deoxyglucose uptake in human myotubes. Cells were depleted of serum in DMEM containing 5,5 mM glucose (low glucose) for 16 h and incubated in the absence or presence of insulin for 40 min at 37°C, and then 2-deoxyglucose uptake was measured using a colorimetric assay kit from Bio Vision. **A)** 2-DG uptake performed in control myotubes (T5) in the absence or presence of increasing concentration of insulin (1 μM, 100 nM and 10 nM). **B)** 2-DG uptake performed in control, DM1 and DM2 myotubes (T5) in the absence or presence of 10 nM insulin. Bars represent standard deviation.

2.3 Insulin signaling activation

When insulin binds its receptor, the activated IR tyrosine kinase phosphorylates several intracellular substrates, starting a very complex cascade of biochemical signals with two major signalling pathways: one mediated by the activation of PI3K and the other by the activation of RAS (Belfiore et al., 2009). To investigate the response to insulin stimulation, 5 days differentiated myotubes (T5) have been treated from 0 to 30 minutes with 10 nM insulin as described in figure 1 and the activation of several proteins involved in PI3K pathway has been analyzed by western blot. As shown in figure 4, western blot analysis confirmed the glucose uptake results. Indeed, the analysis of AKT, p70 and GSK3β activation has shown that control myotubes at five days of differentiation are responsive to 10 nM insulin despite the higher expression of the fetal isoform of the insulin receptor. On the contrary, DM1 and DM2 myotubes exhibited a lower regulation of these proteins compared to controls (figure 4, 2). In particular, AKT phosphorylation in control myotubes increased after 5 minutes of insulin stimulation. This increase was lower in DM myotubes. However, while in DM1 muscle cells an increase in AKT activation was still evident at 5 minutes of insulin stimulation, in DM2 cells insulin did not seem to have a positive effect on AKT activation. In control myotubes was clearly evident also an activation of p70, with a maximum phosphorylation at 15 minutes of insulin stimulation. Again, this increase was less evident in DM1 muscle cells, while in DM2 myotubes the activation of this protein did not change during insulin stimulation. Finally, while in control myotubes insulin induced a down-regulation of the active GSK3β isoform (Y216-GSK3β), DM myotubes showed an increase in the expression of this GSK3β isoform during the 30 minutes of stimulation. These results on the insulin signaling activation clearly demonstrated that post receptor signaling abnormalities might contribute to DM insulin resistance regardless the alteration of IR splicing.

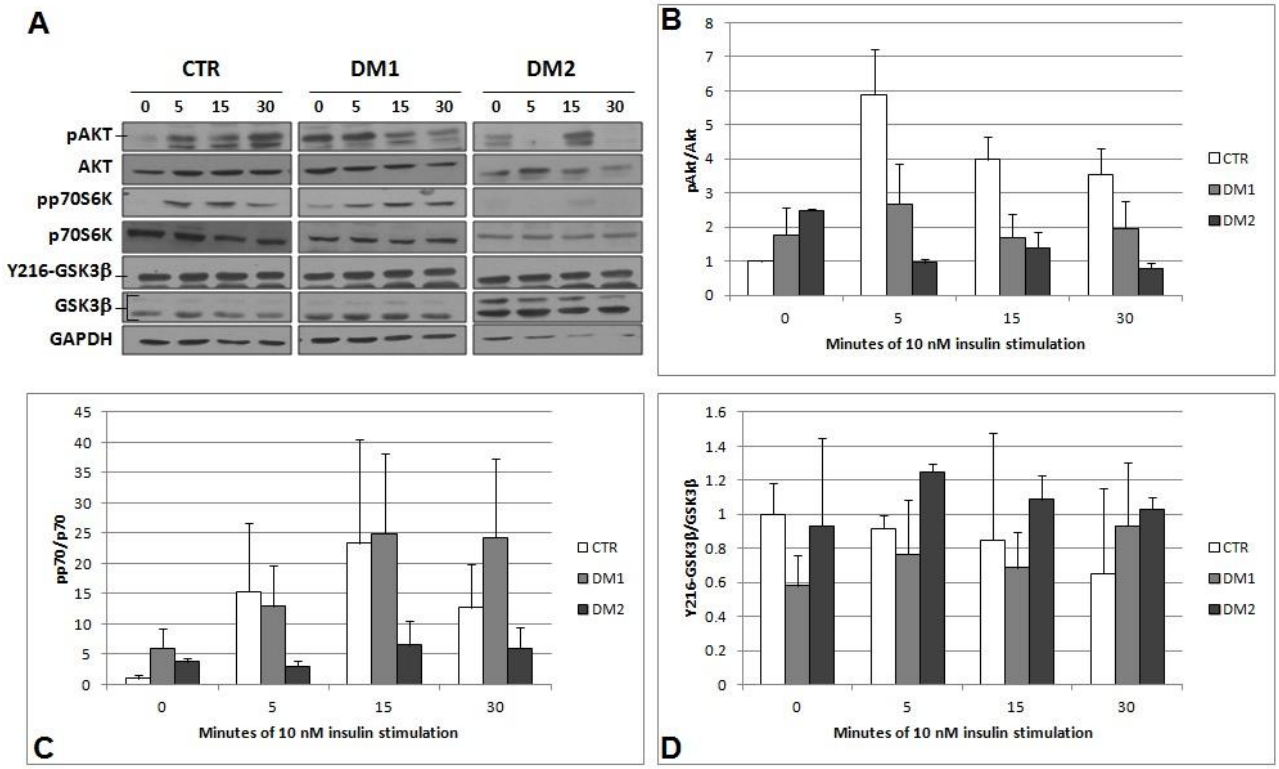

Figure 4: Insulin signaling activation. **A)** Western blot analysis of the activation of some proteins involved in the insulin pathway. Myotubes at 5 days of differentiation were depleted of serum in DMEM containing 5,5 mM glucose (low glucose) for 16 h and incubated in the absence or presence of 10 nM insulin for 0 to 30 min at 37°C. **B)** Activation of AKT in control, DM1 and DM2 myotubes. **C)** Activation of p70S6K in control, DM1 and DM2 myotubes. **D)** Activation of GSK3β in control, DM1 and DM2 myotubes. Bars represent standard deviation.

2.4 Cytoskeleton organization

In order to investigate the molecular mechanisms that induce insulin resistance in skeletal muscle in both DM1 and DM2 patients, we decided to analyze cytoskeleton organization in DM1 and DM2 muscle cells. Indeed, it is known that the binding of insulin to its receptor activates a complex pathway culminating in the translocation of the glucose transporter GLUT4 into the plasma membrane and the cytoskeleton provides a scaffold for intracellular transport such as the movement of vesicles and organelles. The importance of identifying the molecular mechanisms by which cytoskeletal elements contribute to GLUT4 translocation is underscored by recent studies that highlight defects in actin dynamics in conditions of insulin resistance (JeBailey et al., 2007; Zaid et al, 2008). However, there are few studies regarding cytoskeleton reorganization in response to insulin and no studies have been performed on human muscle cells. Since it has been observed that actin filaments seems to play a role in GLUT4 translocation in L6 myotubes, we firstly analyzed actin remodelling in control and DM myotubes. Phalloidin staining during 60 minutes of insulin stimulation did not show any difference in control and DM myotubes. Indeed, remodelling

in actin cytoskeleton was present in few myotubes and with no direct correlation with insulin stimulation (figure 5). These results did not confirm those reported by other authors for actin dynamics in L6 and C2C12 myotubes, where actin ruffles formation was evident upon insulin stimulation. However, these differences could be explained by the different cell line and/or by the different insulin concentration used (Khayat et al., 2000; Bisht and Dey, 2008; Liu et al., 2013).

We then decided to analyze if a reorganization in microtubules network could play a role in GLUT4 translocation after insulin stimulation, since microtubules serve as tracks for directed intracellular transport. Microtubules are hollow cylindrical polymers that are assembled from heterodimers composed of α- and β-tubulin. Confocal analysis of α-tubulin immunostaining at 0, 5, 15, 30 and 60 minutes of 10 nM insulin stimulation did not show any remodelling of microtubule network both in control and DM myotubes (data not shown). However, it is well known that microtubule network is involved in insulin-stimulated plasma membrane translocation of GLUT4 (Fletcher et al., 2000; Liu et al., 2013). Therefore, our fluorescence analysis of α-tubulin could have not allowed us to observe the expected microtubule remodelling. For these reasons, we decided to analyze another member of the tubulin family, γ-tubulin, which is involved in the formation of new microtubule polymers ("nucleation") that could occur in myotubes during insulin stimulation. The analysis of γ-tubulin staining in control myotubes (T5) has shown that there is an insulin dependent increase in microtubule nucleation that reach its highest level after 15 minutes of 10 nM insulin stimulation (figure 6, 7A). In particular, this modulation in microtubule nucleation was associated with an insulin-dependent increase in the percentage of cytoplasmic γ-tubulin spots that was evident in every myotube analyzed (15% of cytoplasmic spots at 0 minutes *vs* 37% of cytoplasmic spots at 5 minutes *vs* 12% of cytoplasmic spots at 60 minutes) (figure 6, 7B). The results obtained in control myotubes confirmed the hypothesis that microtubule network is involved in insulin action in human muscle cells as well as in other muscle cell lines (Fletcher et al., 2000; Liu et al., 2013). Interestingly, γ-tubulin staining has shown that DM muscle cells exhibit a defective microtubule reorganization after insulin stimulation. Indeed, the number of γ-tubulin spots per myotubes did not increase during 60 minutes of stimulation in DM muscle cells, where on the contrary the number of cytoplasmic spots decreased during insulin stimulation (figure 6,7A,7B). Moreover, DM myotubes exhibit a 2 fold increase in microtubule nucleation before insulin stimulation as compared to controls. These results were confirmed by western blot, were control myotubes showed a 3 fold increase in γ-tubulin expression during 30 minutes of insulin stimulation, while on the contrary DM1 and DM2 cells exhibited a decrease in its expression (figure 7C, 7D). Taken together these results seem indicate that DM muscle cells have a global alteration in microtubule nucleation and stabilization. This alteration is consistent with the observed increased activation of ERK1/2 and

GSK3β (figure 4D, 7C, 7E, 7F). Indeed, polymerization and stability of microtubules is regulated by a number of microtubule associated proteins (MAPs) and moreover ERK and GSK3β have been shown to phosphorylate and regulate the binding activity to microtubules of these proteins. ERK1/2 (extracellular signal-regulated kinases) belong to the family of MAPK (mitogen-activated protein Kinases) and are strongly activated by growth factors, cytokines, osmotic stress, and microtubule disorganization (Lewis et al., 1998). Moreover, once activated, they phosphorylate numerous substrates in all cellular compartments, including cytoskeletal proteins (Chen et al., 2001). GSK3β is a constitutively active protein kinase whose activity is inhibited by phosphorylation upon insulin stimulation. Interestingly, type 2 Diabetes (T2D) is strongly associated with a decrease in insulin-stimulated glycogen synthesis along with increased GSK3β protein levels in the muscle (Bogardus et al., 1984; Shulman et al., 1990; Kelley et al., 1996; Cline et al., 1999).

These data indicated that microtubule abnormalities might contribute to insulin resistance observed in DM myotubes. However, further analysis are necessary to understand if an alteration in other cytoskeletal proteins may be involved in the molecular mechanism that induce insulin resistance in myotonic dystrophy.

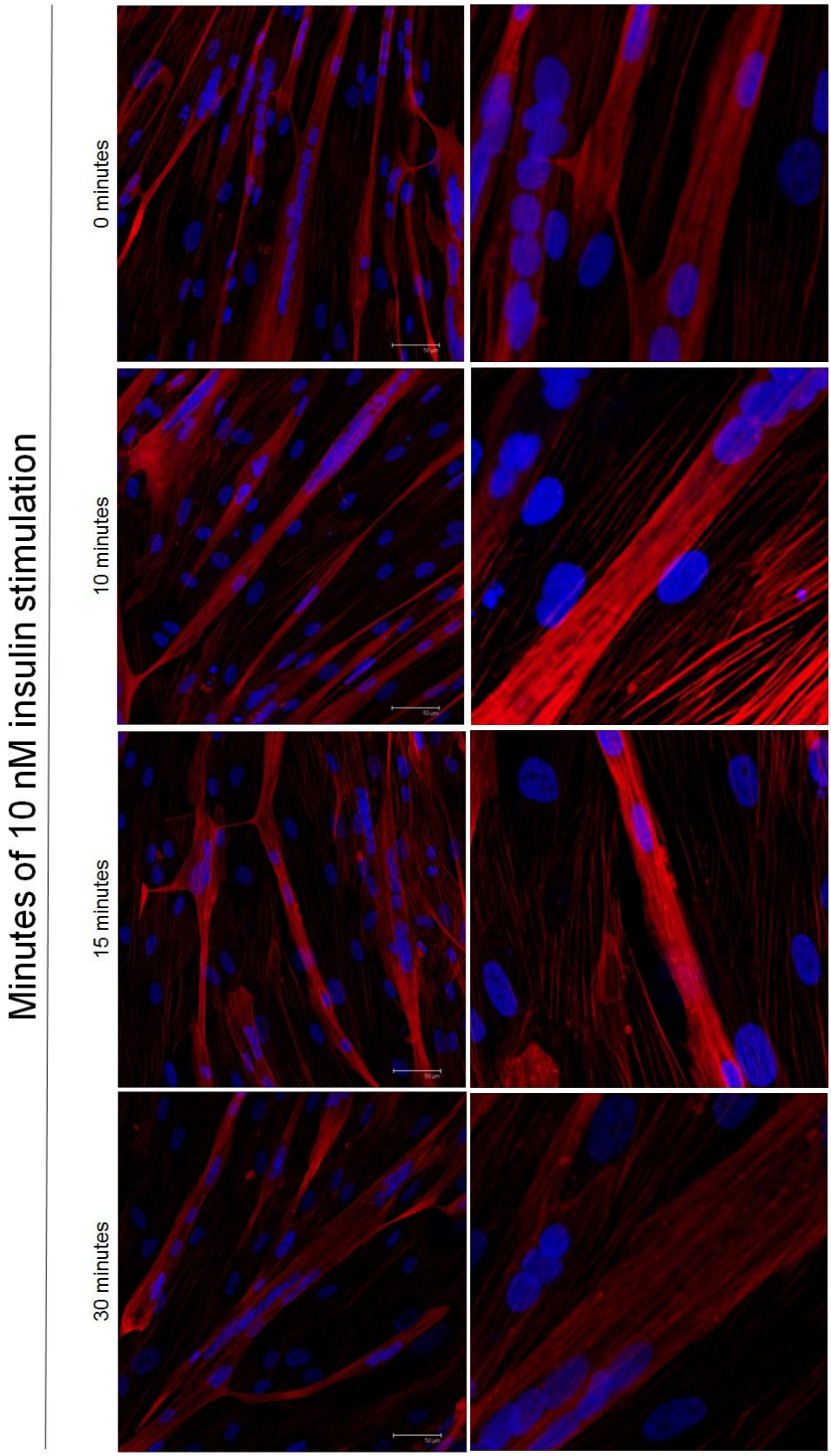

Figure 5: Actin dynamics during insulin stimulation. Myotubes (T5) were depleted of serum in DMEM containing 5,5 mM glucose (low glucose) for 16 h, incubated in the absence or presence of 10 nM insulin for 0 to 60 min at 37°C and then fixed, permeabilized and stained for actin (phalloidin, red) and for nuclei (DAPI, blu). The images are representative of actin remodelling in control muscle cells. Scale bar 50 µM.

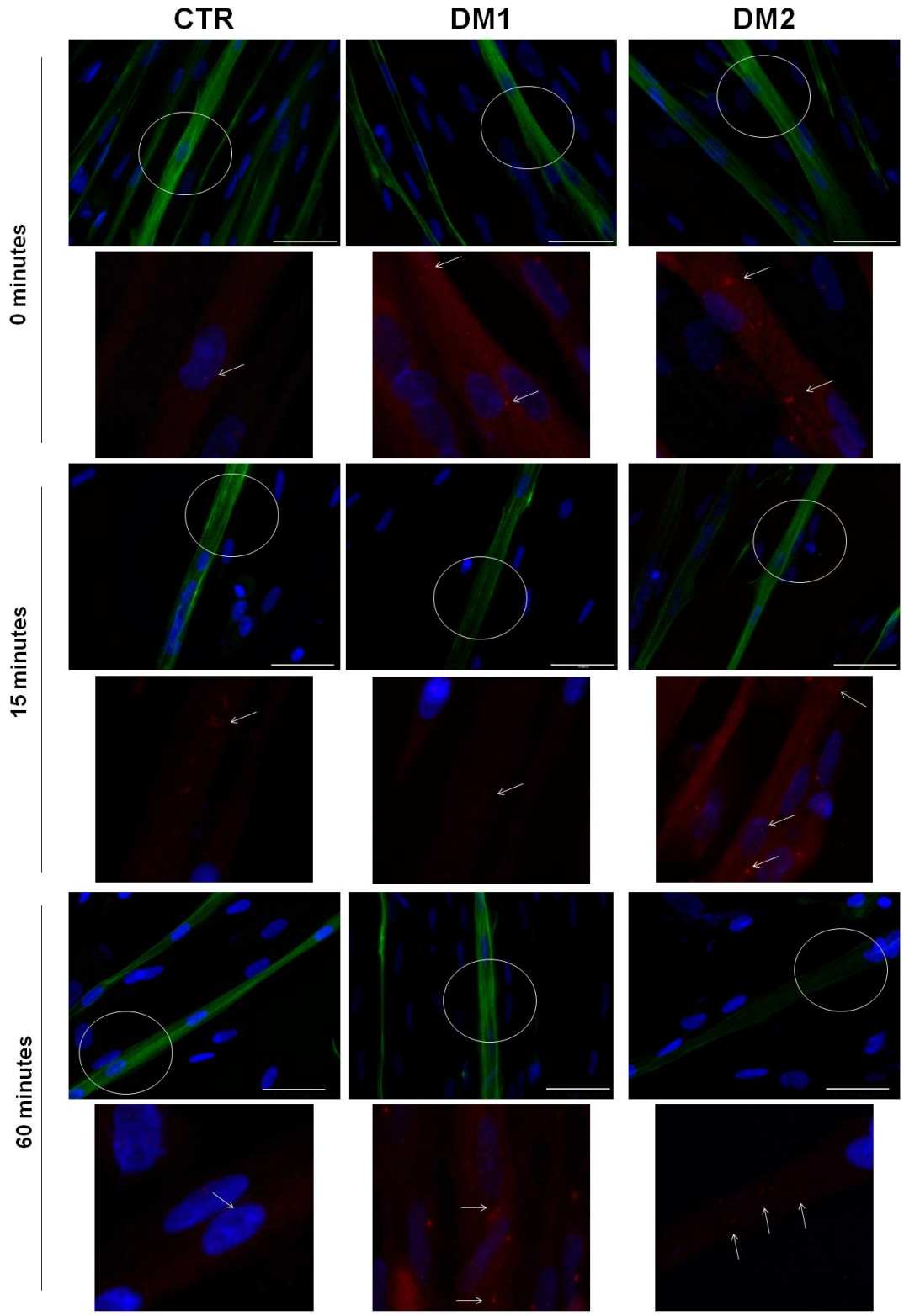

Figure 6: Microtubule nucleation during insulin stimulation. Myotubes (T5) were depleted of serum in DMEM containing 5,5 mM glucose (low glucose) for 16 h, incubated in the absence or presence of 10 nM insulin for 0 to 60 min at 37°C and then fixed, permeabilized and double stained for γ tubulin (red, enlargements), fast myosin (green) and for nuclei (DAPI, blu). The images are representative of microtubules nucleation in control (left panel), DM1 (central panel) and DM2 (right panel) muscle cells after 0, 15 and 60 minutes of 10 nM insulin stimulation. Scale bar 200 μM.

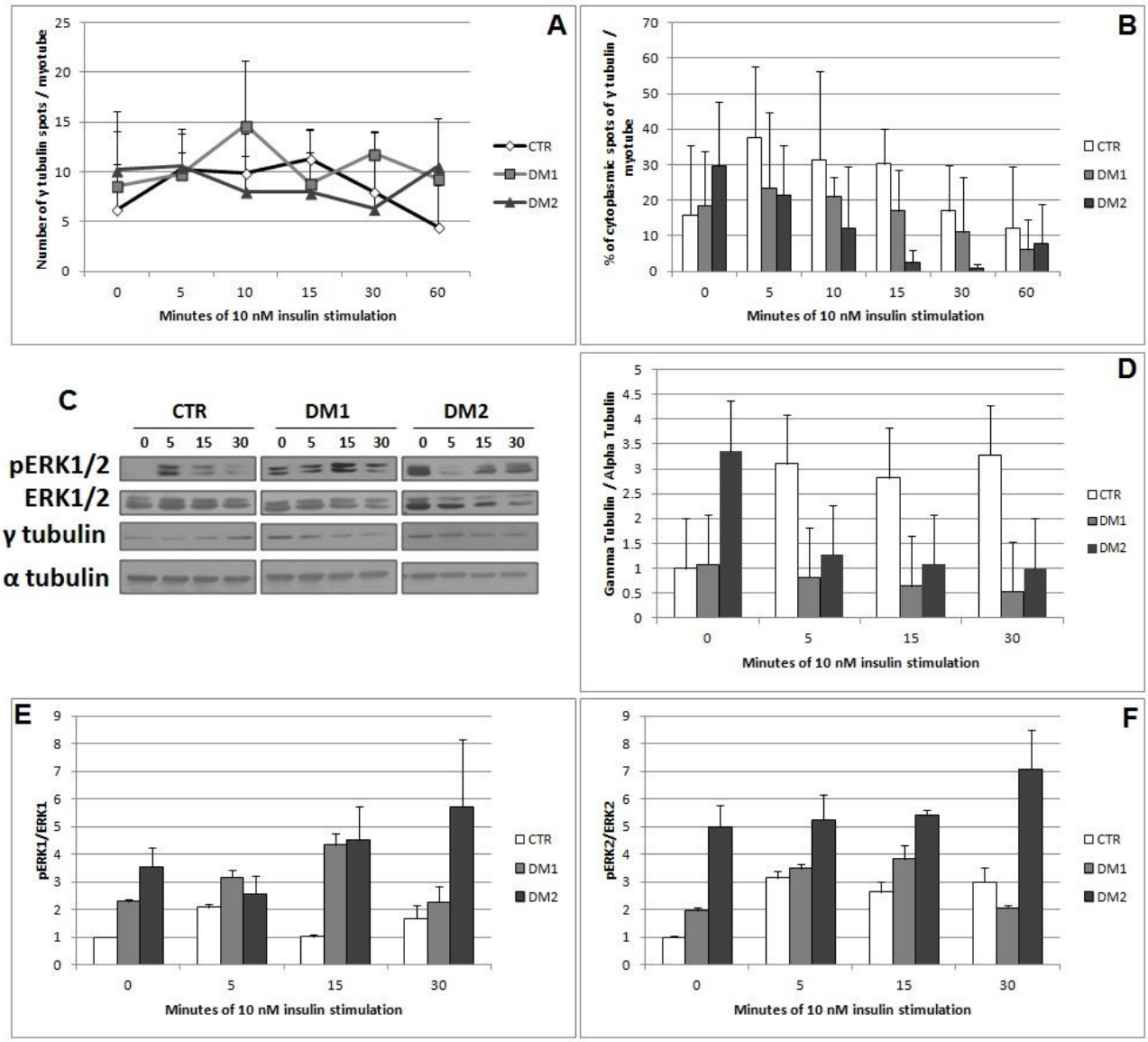

Figure 7: Involvement in microtubule network in insulin signaling. **A)** Number of γ-tubulin spots/myotubes. Number of γ-tubulin spots counted in 5 myotubes per time point in control, DM1 and DM2 coltures. **B)** Percentage of cytosolic γ-tubulin spots counted in 5 myotubes per time point in control, DM1 and DM2 cultures. **C)** Western blot analysis of the activation ERK1/2 and of the expression of γ-tubulin in control, DM1 and DM2 myotubes. **D)** Expression of γ-tubulin during 30 minutes of 10 nM insulin stimulation in control, DM1 and DM2 myotubes. **E-F)** Activation of ERK1 and ERK2 during 30 minutes of 10 nM insulin stimulation in control, DM1 and DM2 myotubes. Bars represent standard deviation.

2.4 GLUT4 translocation

In skeletal muscle, stimulation by insulin results in a significant increase in glucose uptake, which is mediated by the glucose transporter GLUT4, one member in a family of six glucose transporter proteins (Bell et al., 1993). Subcellular fraction analysis of both adipose cell and skeletal muscle cells has shown that upon insulin stimulation, GLUT4 appears to translocate from an intracellular storage compartment to the plasma membrane (Birnbaum 1992; Holman and Cushman, 1994). Confocal analysis of 5 days differentiated myotubes (T5) immunostained for GLUT4 has

shown that in control cells, at basal level, the punctinate staining of GLUT4 was mainly localized in the perinuclear region (red arrows, figure 8). Moreover, additional punctinate staining was evident along the axes of control myotubes. These evidences are consistent with what other authors reported in C2C12 muscle cells (Ralston and Ploug, 1996). After 15 minutes of insulin stimulation, a slight decrease in perinuclear staining associated with the formation of additional cytoplasmic aggregates was evident in every control myotube analyzed (yellow arrows, figure 8). Finally, after 30 minutes of 10 nM insulin stimulation, GLUT4 appeared to be uniformly expressed in sarcoplasm of control muscle cells. These changes in GLUT4 localization at different time points of insulin stimulation seemed to indicate that in control myotubes insulin induced a translocation of GLUT4-storage vesicles. On the contrary, in both DM1 and DM2 differentiated myotubes, this movement of GLUT4 vesicles was not clearly evident. Indeed, in DM1 cells GLUT4 appeared to be uniformly express in the sarcoplasm of myotubes at every time point analyzed (figure 8). Interestingly, DM2 myotubes showed an evident perinuclear staining at basal level as observed in controls. However, the formation of cytoplasmic aggregates was already evident in myotubes not stimulated with insulin (yellow arrows). On the contrary after 15 and 30 minutes of insulin stimulation GLUT4 localization appeared to be uniformly expressed in DM2 cells. These results seemed to indicate that the translocation of GLUT4-storage vesicles is impaired in DM1 and DM2 myotubes and these alterations are consistent with the global alteration in microtubule nucleation observed in these cells. However, our analysis did not allow us to detect the plasma membrane GLUT4 localization either in control and in DM myotubes at the time points of insulin stimulation considered. For this reason, we are currently analyzing the subcellular distribution of GLUT4 by western blot after subcellular fractionating as previously reported by Yonemitsu et al. (2001). The results will finally let us understand if a defect in the translocation of GLUT4 from the perinuclear region to the plasma membrane could be the reason behind the impaired increase in glucose uptake observed in DM myotubes (figure 3). Moreover, in DM1 cells immunofluorescence analysis have shown an evident nuclear staining of GLUT4 that is less evident in control and DM2 samples. The nuclear localization of GLUT4 has never been investigated by others, and currently we are performing nuclear and cytoplasmic protein separation to analyze the nuclear expression of this protein in control, DM1 and DM2 myotubes.

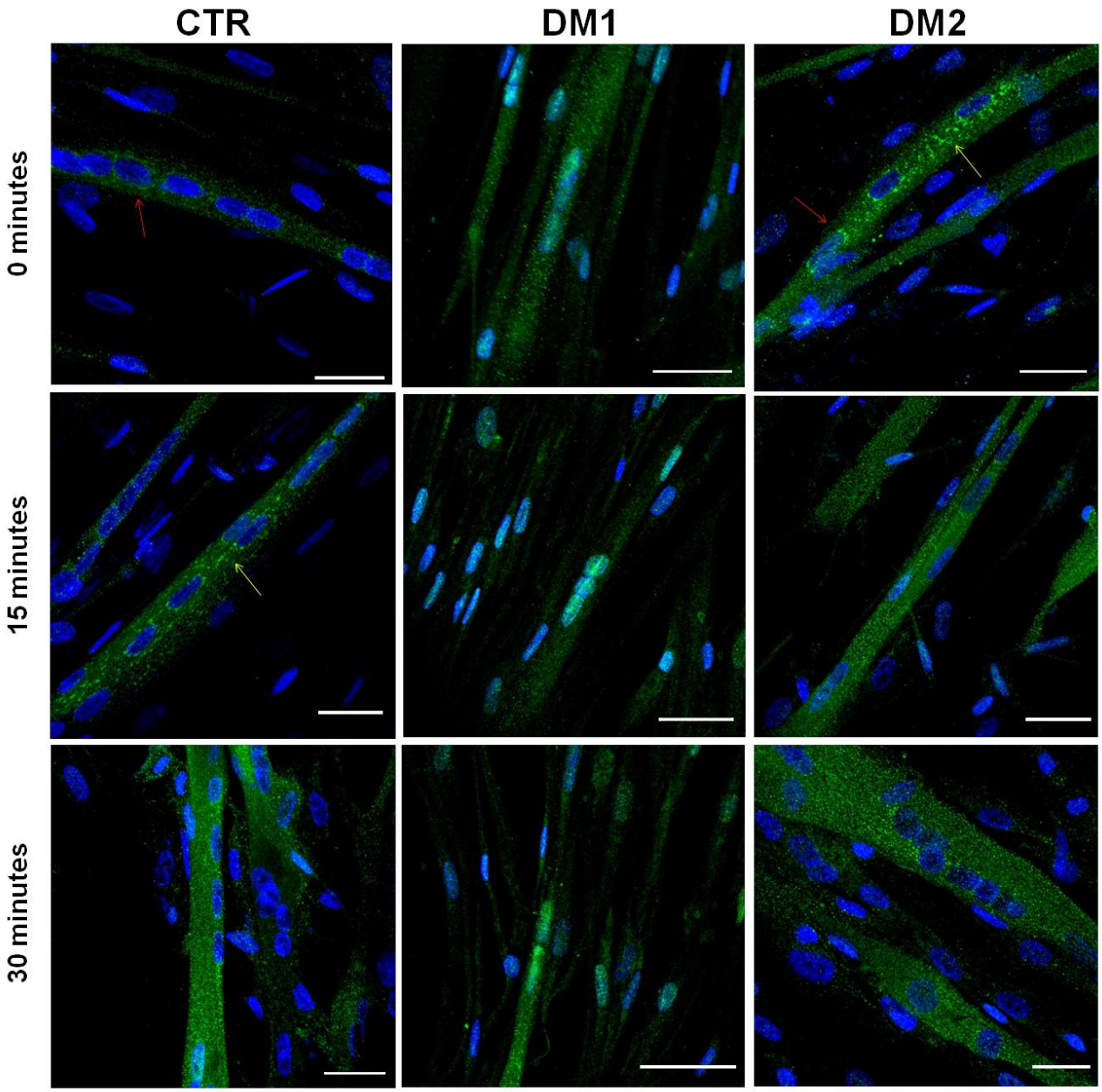

Figure 8: Immunofluorescence localization of GLUT4 in 5 days differentiated control, DM1 and DM2 myotubes. Myotubes (T5) were depleted of serum in DMEM containing 5,5 mM glucose (low glucose) for 16 h and incubated in the absence or presence of 10 nM insulin for 0 to 60 min at 37°C, and then fixed, permeabilized and stained for GLUT4 (green) and for nuclei (DAPI, blu). The images are representative of GLUT4 translocation in control (left panel), DM1 (central panel) and DM2 (right panel) muscle cells after 0, 15 and 30 minutes of 10 nM insulin stimulation. Arrows indicate the presence of perinuclear staining (yellow arrows) and of cyoplasmic aggregates (red arrows). Scale bar 20 μM.

3. Conclusions and future prospects

DMs are characterized by metabolic dysfunctions such as insulin resistance, hyperinsulinemia, hypertriglyceridemia, increased fat mass, and a fourfold higher risk of developing Diabetes Mellitus type 2 (T2DM) (Meola 2000). In literature there are few studies aimed to clearly define the

mechanisms underlying insulin resistance in DM. Savkur et al. (2001) suggested that splicing alteration of insulin receptor (IR) may play a role in peripheral insulin resistance. This splicing alteration leads to a higher expression of the fetal isoform A (IR-A, lacking exon 11) than the isoform B (Savkur et al., 2001). IR-B differs from IR-A by the inclusion of exon 11 that leads to a higher insulin signalling capability. This study has been the first one that elucidated the molecular mechanisms that induce insulin resistance in DM myotubes. In particular, our results indicated that post receptor signalling abnormalities might contribute to DM insulin resistance regardless the alteration of IR splicing. However, further investigations will be necessary to understand whether these alterations may contribute to the histopathological changes observable in skeletal muscle. The results will lead to the identification of novel therapeutic approaches to prevent these features of the disease. Metformin is now considered the first line drug for insulin resistance diseases, including DM, and it increases glucose uptake in muscle through an insulin-indipendent pathway (Kouki et al., 2005). In the recent years, some important component of many foods like Resveratrol, Betaine and Carnitine have found to be insulin mimetic compounds since they activate insulin/IGF1 signalling pathway leading to hypertrophic effects of C2C12 murine muscle cells (Montesano et al., 2013; 2015; Senesi et al., 2013). Moreover, Resveratrol has been shown to act on skeletal muscle metabolism and function and recently it has been reported to influence alternative splicing of pre-mRNA in DM1 fibroblasts where it enhanced the inclusion of exon 11 of the *IR* gene, providing a justification of resveratrol as a leading compound to improve glucose tolerance in DM1 (Takarada et al., 2015). For these reasons it could be interesting to study the effects of resveratrol, betaine and carnitine on insulin resistance and skeletal muscle atrophy in DM patients as alternative drugs to metformin since they are natural insulin mimetic compounds that might have lower side effects.

In conclusion, developing therapies for the prevention and treatment of insulin resistance condition and muscle atrophy process will enhance the quality of life of patients affected by myotonic dystrophies.

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